The Role of Steroidogenic Factor-1 (SF-1) in Transcriptional Regulation of the Gonadotropin-Releasing Hormone (GnRH) Receptor Gene.

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

SUMMARY

The GnRH receptor is a G-protein-coupled receptor in pituitary gonadotrope cells. Binding of its ligand, GnRH, results in synthesis and release of gonadotropin hormones luteinizing hormone (LH) and follicle stimulating hormone (FSH). Steroidogenic factor 1 (SF-1), a transcription factor, binds to specific sites in the promoter region of gonadotropin genes, and thus regulates transcription of these genes. The promoter region of the GnRH-receptor gene contains two SF-1-like binding sites, one at -14 to -8 (site 1) and another at -247 to -239 (site 2), relative to the methionine start codon. The role played by these two SF-1-like sites in basal transcription of the mouse GnRH receptor (mGnRH-R) gene in a pituitary precursor gonadotrope cell line, αT3 cells, was the first area of investigation during this study. Luciferase reporter constructs containing 580 bp of mGnRH-R gene promoter were prepared, where SF-1-like sites were either wildtype or mutated. Four such constructs were made, i.e. wildtype (LG), site 1 mutant (LGM1), site 2 mutant (LGM2) and mutated site 1 plus site 2 (LGM1/2). These constructs were transfected into αT3 cells to determine the effect of mutations of sites 1 and/or 2 on the basal expression of the mGnRH-R gene. Mutation of either site 1 or site 2 had no effect on basal expression of the mGnRH-R gene. It was found that only upon simultaneous mutation of both sites 1 and 2, a 50% reduction in basal transcription took place. The implications of this is that SF-1 protein seems to only require one intact DNA-binding site, to mediate basal transcription of the mGnRH-R gene, suggesting that these two sites lie in close proximity during basal transcription. The effect of the protein kinase A (PKA) pathway on the endogenous mGnRH-R gene was also investigated by incubating non-transfected αT3 cells with the PKA activators, forskolin and 8-Br-cAMP. Similar incubations were also performed on the wild type and mutated site 1 constructs transfected into pituitary gonadotrope αT3 cells. It was found that forskolin and 8-Br-cAMP were able to increase endogenous mGnRH-R

mRNA levels in a concentration-dependent fashion, showing that endogenous GnRH receptor gene expression is stimulated via a protein kinase A pathway. Similar results were obtained with the wildtype promoter construct, showing that the protein kinase A pathway stimulates transcription of the promoter. This effect was only seen with wild type and not with the mutated site 1. These results are consistent with a role for a SF-1-like transcription factor in mediating the protein kinase A effect via binding to the site 1 at position –14 in the GnRH receptor gene. A separate investigation was performed to determine whether 25-hydroxycholesterol (25-OHC) is a ligand for SF-1, by incubating αT3 cells transfected with the various constructs with 25-OHC. Results show a dose-dependant response, with an increase in gene expression at 1 μM and a decrease at higher concentrations, for both mutant and wild type constructs. This suggests that, if SF-1 is indeed the protein binding to sites 1 and 2, then 25-OHC is not a ligand for SF-1 protein in αT3 cells and that the effect of 25-OHC on the mGnRH-R gene is not mediated via site 1. The results indicate that these decreases of expression at the higher concentrations may be due to cytotoxic effects. Towards the end of the study the laboratory obtained a luminoskan instrument with automatic dispensing features. Optimisation studies on the luciferase and β-Gal assays were performed on the luminoskan in a bid to decrease experimental error. It was found that automation of these assays resulted in a decrease in experimental error, showing that future researchers could benefit substantially from these optimisation studies.

OPSOMMING

Die GnRH reseptor is 'n G proteïen-gekoppelde reseptor in pituitêre gonadotroopselle. Binding van die ligand, GnRH, lei tot die sintese en vrystelling van die gonadotropien hormone, luteïniserende hormoon (LH) en follikel stimulerende hormoon (FSH). Steroidogeniese faktor-1 (SF-1) is 'n transkripsie faktor wat aan spesifieke areas in die promotergebied van die gonadotropien hormone bind, en dus transkripsie van hierdie gene reguleer. Die promotergebied van die GnRH reseptor geen bevat twee SF-1 bindings areas, een by -14 to -8 (area 1) asook by -247 to -239 (area 2), relatief to die metionien beginkodon. Die rol wat hierdie twee SF-1 areas speel in basale transkripsie van die muis GnRH reseptor (mGnRH-R) geen in 'n pituïtêre voorloper gonadotroop sellyn, αT3 selle, was die eerste gebied van ondersoek gedurende hierdie studie. Plasmiede bestaande uit die 580 basispaar mGnRH-R promoter verbind aan 'n lusiferase geen is vervaardig, waar SF-1-soortige areas enersyds onveranderd gelaat is, of gemuteer is. Vier sulke plasmiede is vervaardig, nl. onveranderd (LG), area 1 mutant (LGM1), area 2 mutant (LGM2) en gemuteerde area 1 plus area 2 (LGM1/2). Hierdie plasmiede is gebruik om αT3 selle te transfekteer om die effek van mutasies van areas 1 en/of 2 op die basale ekspressie van die mGnRH-R geen te ondersoek. Daar is gevind dat mutasies van areas 1 of 2 geen effek op basale ekspressie op die bogenoemde geen gehad het nie. Slegs tydens gelyktydige mutasie van areas 1 en 2 het 'n 50% vermindering in basale transkripsie plaasgevind. Die implikasies hiervan is dat die SF-1 proteïen blykbaar slegs een volledige DNA-bindingsarea benodig om basale transkripsie van die mGnRH-R geen te reguleer. Dit wil dus voorkom of hierdie twee areas baie na aan mekaar geposisioneer is tydens basale transkripsie. Die effek van die proteïen kinase A (PKA) roete op die natuurlike mGnRH-R geen is ook ondersoek tydens inkubasie van nie-getransfekteerde αT3 selle met die PKA akiveerders, forskolin en 8-Br-cAMP. Soortgelyke inkubasie is ook gedoen op die onveranderde en gemuteerde area 1 plasmiede wat in αT3 selle getransfekteer is. Daar is gevind dat forskolin en

8-Br-cAMP daarin geslaag het om die natuurlike mGnRH-R geen mRNA vlakke op 'n konsentrasie-afhanklike wyse te vermeerder. Hierdie resultaat dui daarop aan dat die natuurlike mGnRH-R geen se ekspressie gestimuleer kan word via 'n proteïen kinase A roete. Soortgelyke resultate is verkry met die onveranderde promoter plasmied en dit wys ook daarop dat proteïen kinase A transkripsie deur die promoter kan stimuleer. Hierdie effek was slegs aanwesig met die onveranderde en nie met die gemuteerde area 1 plasmied nie. Die resultate stem ooreen met 'n rol vir SF-1 transkripsie faktor in die regulering van proteïen kinase A effek deur middel van binding aan die area 1 by posisie -14 in die GnRH-R geen. 'n Afsonderlike ondersoek is gedoen om vas te stel of 25-hidroksiecholesterol (25-OHC) 'n ligand vir SF-1 is deur getransfekteerde αT3 selle met 25-OHC te inkubeer. Resultate toon 'n dosis-afhanklike respons met 'n verhoging in geen ekspressie by 1 μM en 'n verlaging met hoër konsentrasies vir beide onveranderde en gemuteerde plasmiede. Dit impliseer dat, indien SF-1 wel die faktor is wat aan areas 1 en 2 bind, 25-OHC nie die ligand vir SF-1 proteïen in αT3 selle is nie en dat die effek van 25-OHC op die mGnRH-R geen nie gereguleer word via area 1 nie. Die verlaging in ekspressie gevind by die hoër konsentrasies is dalk die gevolg van sitotoksiese effekte. Teen die einde van die studie het die laboratorium luminoskan toerusting met outomatiese pipettering verkry. Optimiseringstudies van die lusifirase en β-Gal toetse is met die luminoskan gedoen in 'n poging om eksperimentele foute te minimaliseer. Daar is gevind dat outomatisering van hierdie toetse wel gelei het tot 'n verlaging in eksperimentele foute. Toekomstige navorsers kan dus grootliks voordeel trek uit hierdie optimiseringstudies.

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ABBREVIATIONS

25-OHC	25-hydroxycholesterol
AP-1	activating protein-1
cAMP	cyclic adenosine 3',5'-monophosphate
Ci	Curie
COUP-TF	chicken ovalbumin upstream promoter-transcription factor
CRE	cAMP response element
CREB	CRE binding protein
DAG	diacylglycerol
DAX-1	dosage-sensitive sex reversal-adrenal hypoplasia congenita critical region on the X chromosome
DEPC	diethylpyrocarbonate
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
dNTP	deoxynucleoside triphosphate
dpm	disintegrations per minute
EDTA	ethylediaminetetra-acetic acid tetrasodium salt
FCS	fetal calf serum
FSH	follicle stimulating hormone
GDP	guanidine diphosphate
GnRH	Gonadotropin-releasing hormone
GnRH-R	Gonadotropin-releasing hormone receptor
GPCR	G-protein coupled receptor
GRAS	GnRH receptor activating sequence
GSE	gonadotrope specific element
HPA	Hypothalamic/Pituitary/Adrenal axis
HPG	Hypothalamic/Pituitary/Gonadal axis
IP_3	inositol triphosphate
LB	Luria-Bertani medium
LH	luteinizing hormone
MAPK	mitogen activated protein kinase

MOPS	morpholinopropanesulfonic acid
OD	optical density
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PKA	protein kinase A pathway
PKC	protein kinase C pathway
SDS	sodium dodecyl sulphate
SF-1	Steroidogenic factor-1
TAE	tris-acetate EDTA
TBE	tris-borate-EDTA
TE	tris-EDTA
UV	ultraviolet

AIM OF THIS STUDY

The aim of this study was as follows: to study the role that steroidogenic factor-1 (SF-1) plays in the transcriptional regulation of the mouse gonadotropin-releasing hormone receptor (mGnRH-R) gene. This was done by using a part of the GnRH receptor gene promoter region that contained two SF-1-like sites (sites 1 and 2), linked to a luciferase reporter construct. Transfection experiments were carried out on a mouse pituitary clonal cell line, i.e. αT3 cells. Some of the important questions that were investigated in this study are outlined below:

1. Does the SF-1-like site 1, in the mGnRH-R promoter region, play a role in basal transcription of this gene?

2. Does the SF-1-like site 2, in the mGnRH-R promoter region, play a role in basal transcription of this gene?

3. Does the simultaneous mutation of both these SF-1-like sites affect the basal transcription of the mGnRH-R gene?

4. Do activators of the protein kinase A (PKA) pathway have an effect on the expression levels of endogenous mGnRH-R mRNA in αT3 clonal cells?

5. Is transcription of the mGnRH-R gene regulated by the actions of the PKA pathway?

6. If the answer to question 5 is yes, then do the SF-1-like sites 1 and 2, mediate this response?

7. Does 25-hydroxycholesterol play a role in transcriptional regulation of the mGnRH-R gene, and if so, is this effect mediated by the SF-1-like site 1?

Additionally the optimisation of some of the experimental procedures, i.e. the luciferase and β-galactosidase assays will also be performed, in order to minimise experimental error in future experiments of this nature.

Previous work done in our laboratory on the mGnRH-R gene, by Janet Hapgood and Carmen Pheiffer involved the identification of the transcriptional start site, mutagenesis of SF-1-like site 1 and preliminary transfection studies. Work being done in our laboratory, by Hanel Sadie, (in parallel to the present thesis) focuses mainly on identifying the proteins in αT3 cells that bind to these SF-1-like sites in the mGnRH-R, using the technique of electrophoretic mobility shift assays (EMSA) or gelshifts. It is hoped, to one day, have a complete picture of mGnRH-R gene regulation and the role that SF-1 protein plays in that regulation in αT3 cells.

CHAPTER 1

INTRODUCTION

1. The role of GnRH receptor in the Hypothalamic-Pituitary-Gonadal (HPG) axis

A.) Characterisation of the GnRH receptor and its second messenger pathways

Gonadotropin-releasing hormone (GnRH), a decapeptide (pyro-Glu-His-Tyr-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) [1] is synthesised by the hypothalamic neurons and released in a pulsatile fashion. In the pituitary it binds to its specific receptor (GnRH receptor, see figure 1.1 for a diagrammatic representation of the GnRH receptor), a member of the G-protein coupled receptor (GPCR) family, and stimulates the release of the gonadotropins luteinizing hormone (LH) and follicle stimulating hormone (FSH) [2] (see figure 1.4). GnRH receptors have also been found in the hypothalamic neurons that release GnRH [3] as well as other regions of the brain including the olfactory system, lateral septum, parasubiculum and the hippocampus [4] where they are thought to act as neurotransmitter receptors linking the endocrine effects of GnRH to reproductive behaviour. The GnRH receptor has recently been identified in the gonadal tissues of rats [5] and it has been postulated that this receptor allows GnRH to have a direct influence on the gonads.

The pituitary is made up of five different cell types i.e. gonadotropes, thyrotropes, lactotropes, somatolactotropes and corticotropes. Of these only gonadotropes express the GnRH receptor on their cell surfaces [6, 7]. The GnRH receptor is a 327 amino acid peptide and forms the distinctive seven transmembrane domains that are associated with the GPCR family [2].

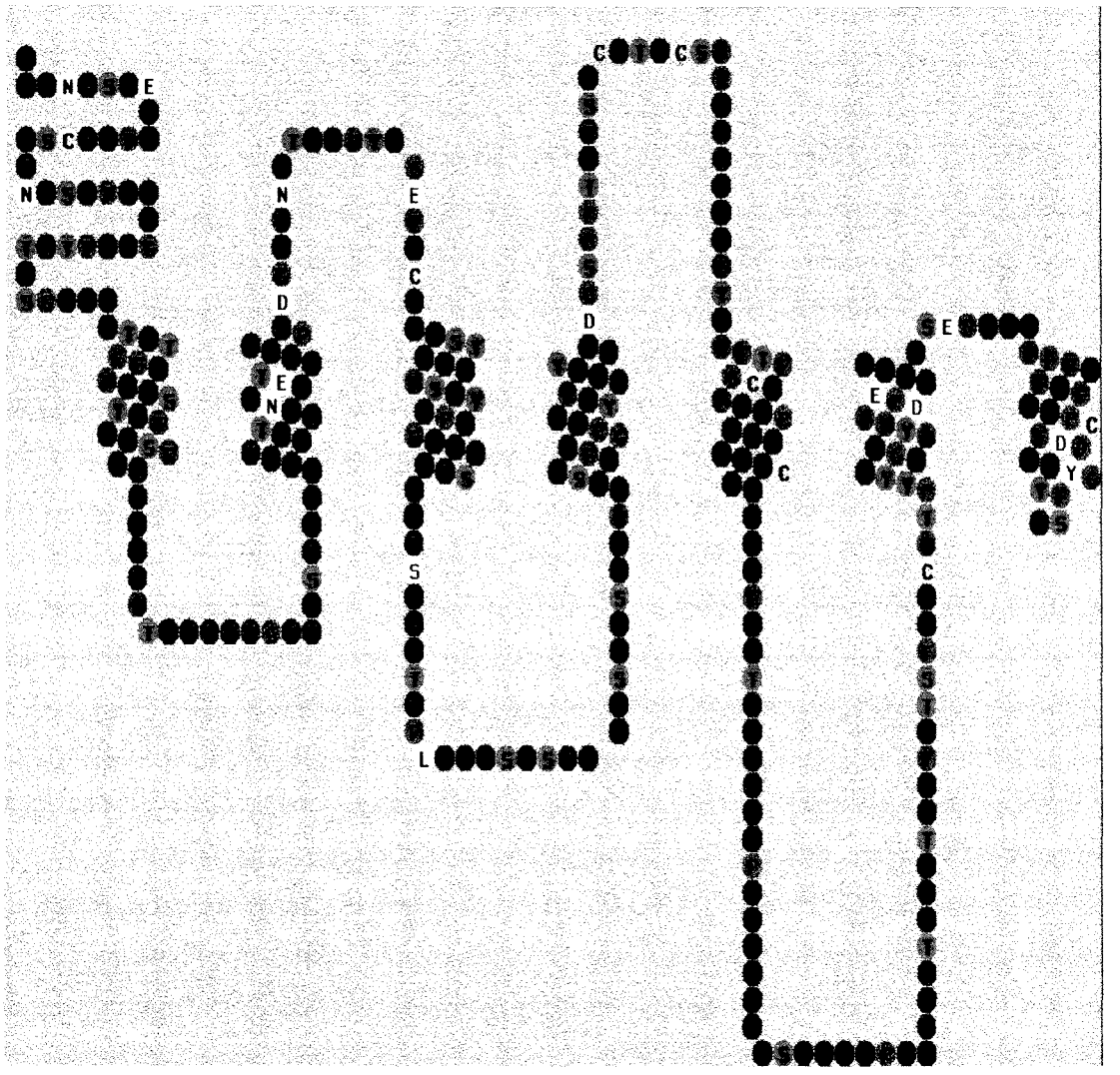


Figure 1.1: The proposed structural configuration of the GnRH receptor in the membrane, showing the seven transmembrane domains as well as the absence of an intracellular C-terminal tail. Figure taken from Horn F., Weare J., Beukers M.W., Hörsch S., Bairoch A., Chen W., Edvardsen Ø., Campagne F., and Vriend G. (1998) GPCRDB: an information system for G protein-coupled receptors. Nucleic Acids Res. 26(1): 277-281 and the G Protein Coupled Receptor DataBase (GPCRDB) at http://www.cmbi.kun.nl/7tm/.

Unlike other members of this family the GnRH receptor lacks an intracellular C-terminal tail [8, 9] (see figure 1.1). Until recently it was thought that the GnRH receptor, like other members of the GPCR family, functions only as a monomeric unit, but recent evidence suggests that the induction of receptor aggregation is sufficient for activation. However, whether this phenomenon of self-aggregation occurs under physiological conditions still remains to be seen [10]. It has also long been the theory that the GnRH receptor was only associated with the $G_{q/11}$ protein, but a recent study in rat gonadotropes by

Stanislaus, D. *et al.* (1998) [11] showed that the GnRH receptor could associate with $G_i\alpha$, $G_s\alpha$ and $G_{q/11}\alpha$ proteins in a single cell, a fact that will be discussed in more detail later on.

After binding of GnRH to its receptor in pituitary gonadotropes, various second messenger pathways are activated to regulate the transcription of the gonadotropin genes as well as its own receptor gene. The most well known second messenger pathway for the GnRH receptor takes place as follows: GnRH binds to its receptor which is coupled to the $G_{q/11}\alpha$ protein. The α subunit of the $G_{q/11}$ protein in turn activates the membrane associated phospholipase C, which produces the second messengers inositol(1,4,5)-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 leads to the mobilisation and influx of Ca^{2+} ions and together with DAG can activate and translocate protein kinase C (PKC) [2, 12]. Recently the picture has been elucidated more clearly and there seems to be a complex series of events that coincide with the above including the activation of phospholipases PLD and PLA_2, release of arachidonic acid and formation of bioactive lipoxygenase products that all play a role in gonadotropin and GnRH receptor gene regulation [13, 14]. An example of the activation of the PKC pathway by the growth factor receptor is shown in figure 1.2. Although it differs slightly from GnRH receptor activation of the PKC pathway, the end result is the same in both cases.

The above however is just the tip of a very complex iceberg. The next steps of the signal transduction pathway involve a series of phosphorylation steps. The Ca^{2+} ion influx into the cell can also activate calmodulin kinase II, which further phosphorylates RAS-GTPase-activating protein (RAS-GAP) [15]. RAS-GAP will in turn lead to the activation of a pathway of serine/threonine protein kinases called the mitogen-activated protein kinase or MAPK pathway. A spate of new evidence [13, 15, 16, 17, 18 and 19] has shown that the MAPK pathway plays a role in the second messenger pathway of the GnRH receptor. RAS-GAP phosphorylates MAPK kinase kinase (MAPKKK or Raf), which in turn phosphorylates MAPK kinase (MAPKK or MEK), which

phosphorylates MAP or ERK (extracellular signal-regulated kinase). ERK is the kinase which can then finally phosphorylate a specific transcription factor, so that transcription of a target gene can take place [14, 20].

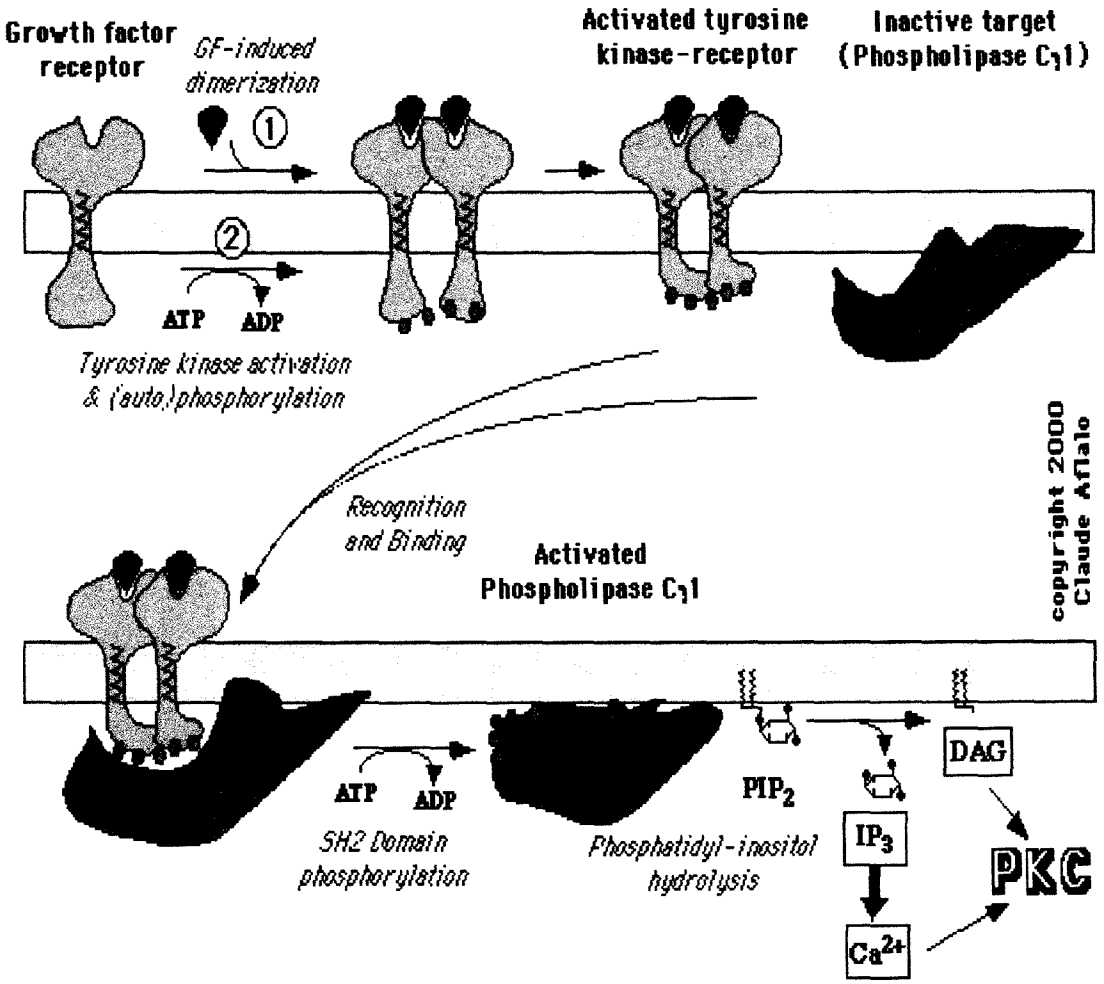


Figure 1.2: A diagrammatic scheme of the activation of the PKC pathway by the growth factor receptor. The abbreviations used are as follows: GF = Growth Factor, IP_3 = inositol 1,4,5-triphosphate, DG = diacylglycerol, cAMP = cyclic 3',5'-adenosine monophosphate, Ca^{2+} = calcium ion, P = phosphate group, PI = phosphoinositol, PIP_2 = inositol 4,5-bisphosphate. Figure taken from http:\\www.bgu.ac.il/~aflaloc/bioca/ and is copyrighted to Claude Aflalo.

The other well known signal transduction pathway involves the protein kinase A (PKA) pathway. Whilst activation of the PKC pathway stems from the GnRH receptor association with the $G_{q/11}\alpha$ protein, the activation of the PKA pathway occurs when GnRH receptor is associated with the $G_s\alpha$ protein. After binding of the ligand the receptor-G protein complex exchanges its bound guanosine

triphosphate (GTP) for guanosine diphosphate (GDP) and thereby activating the membrane associated adenylate cyclase. Activated adenylate cyclase hydrolyses adenosine triphosphate (ATP) to cyclic-adenosine monophosphate (cAMP). cAMP then acts as a second messenger by activating or inhibiting various enzymes or cascades by promoting their phoshorylation or dephosphorylation [21]. A schematic representation of the above mechanism is shown in figure 1.3.

There is evidence that the GnRH receptor utilises the PKA pathway as a second messenger in animals. Borgeat, P. *et al.* (1972) [1] showed that synthetic GnRH causes the accumulation of cAMP in the anterior pituitary gland of male rats. Lui, T-C. *et al.* (1981) [22] also proposed a role for cAMP in GnRH-dependent receptor regulation via the PKA pathway when they found that incubation of anterior primary pituitary cells from ovariectomised rats with cAMP analogues in combination with GnRH caused an increase in the production and release of LH, compared to primary pituitary cells incubated only with GnRH. However when Horn, F. *et al.* (1991) [23] investigated GnRH receptors in an αT3 cell line (immortalised mouse pituitary gonadotropes [24]) and their intracellular responses to GnRH analogues, they found that binding of a GnRH analogue to its receptor did not affect cAMP levels, but instead increased phosphoinositide turnover and protein kinase C translocation, suggesting that in αT3 cells the GnRH receptor preferentially makes use of the PKC rather than the PKA pathway as the second messenger of choice.

An interesting experiment was performed by Kuphal, D. *et al.* (1994) [25] where they stably transfected the GnRH receptor in GH_3 cells (a pituitary lactotrope cell line), now called GGH_3 cells. When these cells were stimulated with GnRH, they found that receptor signalling was dependent on cAMP as a second messenger, indicating that the GnRH receptor can associate with G proteins that activate adenylate cyclase. This is in contrast to Saunders, B.D. *et al.* (1998) [26], who showed that when GGH_3 cells were transfected with

various gonadotropin subunit expression vectors, i.e. α-subunit, LHβ and FSHβ subunit, GnRH-dependent transcription of these constructs was

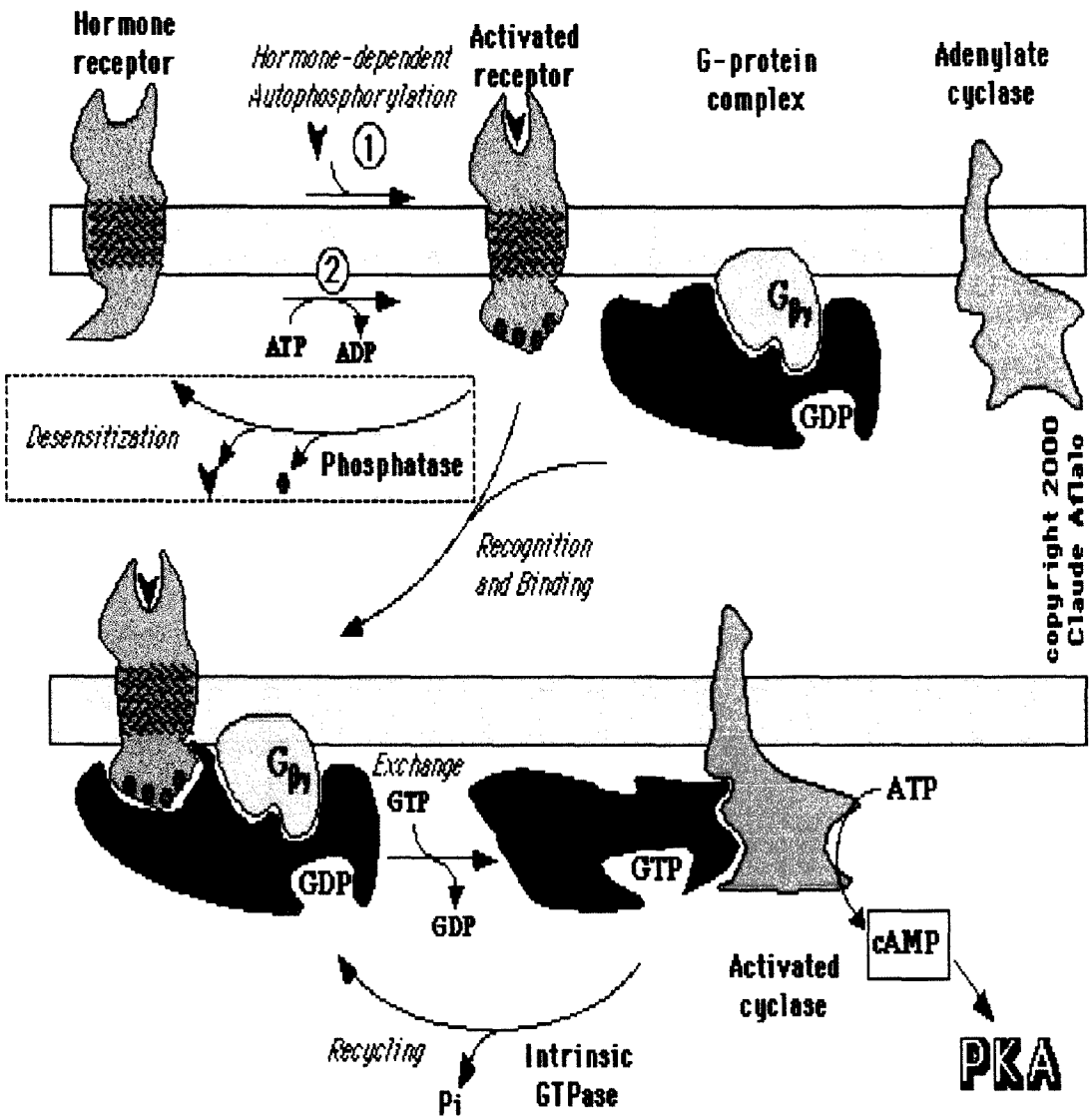


Figure 1.3: A schematic representation of the activation of the PKA pathway. See text for detail. Figure taken from http:\\www.bgu.ac.il/~aflaloc/bioca/ and is copyrighted to Claude Aflalo.

mediated via the PKC pathway and that the PKA pathway played no role. They suggest, however, that the GnRH receptor makes differential use of signal transduction pathways. In fact this divergence in second messenger pathways was found when Weiss, J. *et al.* (1990) [27] investigated the effect of GnRH stimulation on α-, LHβ- and FSHβ subunit mRNA levels in perfused

rat pituitaries. They found a divergence in the regulation of gonadotropin secretory responses related to the mRNA levels which suggest that different signalling pathways may be involved in regulating these two cellular events, i.e. gonodotropin secretion, and changes in mRNA levels of LH and FSH. Further proof of GnRH's ability to activate both the PKA and PKC pathways was shown in recent studies by Lin, X. and Conn, P.M. (1998, 1999) [28, 29] and Han, X. and Conn, M. (1999) [30] done in GGH_3 cells. Their results will be discussed in more detail in section D.

To summarise, it seems that in whole animals or in primary pituitary cells, the signal transduction pathway used by the GnRH receptor is the PKA pathway. In αT3 cells the second messenger of choice is the PKC pathway and in GGH_3 cells, both PKA and PKC are used. These apparent differences can be ascribed to the physiological differences between whole animals, αT3 cells and GGH_3 cells. It may be that the GnRH receptor utilises a different second messenger pathway depending on the cellular and molecular milieu. The differences between whole animals, αT3 cells and GGH_3 cells will be discussed in the next section.

B.) Model systems to study regulation of GnRH receptor gene expression and signalling pathways

There are several model systems which have been used to study regulation of the GnRH receptor gene, including whole animals, transformed cell lines originating from tissue types, primary pituitary cells as well as transgenic animals. When using whole animals, one way to study the effects of the sex hormones is to remove the negative feedback loop of these steroids on the pituitary, by way of castration or ovariectomy of mature rats or mice. Replacement therapy with sex steroids then follows. After an incubation time the animals are sacrificed and levels of GnRH receptor protein and mRNA are measured. Cell lines are mostly derived from tumours induced in

transgenic mice. The two cell lines most frequently used for GnRH receptor studies are αT3 and GGH_3. The αT3 cell line was made by expressing the Simian Virus-40 T-antigen (Tag) oncogene linked to the promoter region from the human α-subunit glycoprotein gene in gonadotrope cells in the anterior pituitary of transgenic mice [24]. The αT3 clonal cell lines derived from these tumours express the mouse α-subunit gene and synthesise and secrete the α-subunit protein, but they are unable to express β-subunit genes. GnRH receptor mRNA and proteins are also found in the same concentration range as in normal pituitary cells. These receptors also respond to GnRH-analogues, with dissociation constants similar to those found naturally [23]. Estrogen-, progesterone-, androgen- and glucocorticoid receptors [31] as well as SF-1 mRNA and protein [7] are all expressed in these cells. On the other hand, the GGH_3 cell line is derived from immortalised lactotrope GH_3 cells from the mouse pituitary, which has been stably transfected with the GnRH receptor and responds to GnRH or its analogues. Upon activation of the receptor, prolactin and growth hormone are released, instead of the gonadotropins FSH and LH, as in normal gonadotrope cells. These cells are useful for studying the second messenger pathways linked to GnRH receptor activation [20, 28, 29, 30]. Primary pituitary cells are not often used as they are difficult to culture and there are often problems with contamination, i.e. the cells are not all pure gonadotropes.

However, the major difference between cell lines and whole animals is the fact that cell lines sometimes act as immature of even fetal gonadotropes, compared to the mature gonadotropes of experimental animals. The clonal cell lines also sometimes lose some of their differentiated functions. Another difference is the presence of multiple other factors in whole animals, e.g. rats, that could work in synergism with steroids and GnRH, but are absent in cell lines. Nonetheless clonal cell lines have proven to be valid and useful model systems to study the effects of GnRH and other sex hormones on the regulation of the GnRH receptor.

Transgenic animals are prepared by expressing the GnRH receptor gene from one animal into another from a different species, e.g. Duval, D.L. *et al.* (2000) [32] created GnRH-R/luciferase transgenic mice containing 9100 bp of the 5'-flanking region of the ovine GnRH receptor gene. The experiments with transgenic mice are probably nearer to the *in vivo* state than the transfection experiments that are usually done in clonal cell lines like αT3 or GGH_3 to determine the importance of *cis*- and *trans*- elements in a certain promoter, but are much more expensive and labour intensive. There is also the problem of introducing a promoter region from a foreign species that may have a different transcriptional regulation and therefore will not be compatible with the transcriptional machinery of the transgenic animal. The added advantage of using a pituitary cell line such as αT3 cells, is the fact that one can look in isolation at each different element at a time and see the direct effects that mutation of these elements have on the activity of the promoter, whilst the changes seen in transgenic animals may be due to other indirect effects. Experiments done in clonal cell lines are by far the norm and have yielded important information which in most cases is consistent with results observed in whole animals.

C.) Regulation of the GnRH receptor by steroid hormones

The GnRH receptor is a very important protein in the regulation of reproduction. The regulation of receptor number and mRNA levels can influence processes that occur further downstream in the hypothalamic-pituitary-gonadal (HPG) axis (see figure 1.4). Regulation of receptor number could occur at multiple levels including transcriptional, RNA stability and transport, translational and post-translational, which could include alterations in degradation and/or recycling of receptor-ligand complexes. During the rat estrous cycle, GnRH receptor numbers on the pituitary gonadotropes vary dramatically in response to a variety of influences, including changes in levels of estrogen, progesterone and increased GnRH secretion (see next

section) [33] as well as members of the TGF-β family of growth and differentiation factors, i.e. activin and inhibin [34]. Results from various experiments, grouped together by model system used, will now be reviewed in more detail.

In animals, estrogen induces a LH surge. Experiments performed by Bauer-Dantoin, A.C. *et al.* (1995) [35], in ovariectomised rats, showed that GnRH receptor mRNA levels are elevated before and throughout this LH surge. After the peak of the LH surge, GnRH receptor mRNA levels rapidly decrease and eventually by 24 hours proestrous end at levels lower than before the LH surge. An interesting observation was the fact that the relative levels of GnRH receptor mRNA levels showed a strong correlation with numbers of GnRH receptor protein molecules [35]. Inhibition of hypothalamic input with pentobarbital treatment blocked the estrogen-induced LH surge and significantly reduced GnRH receptor mRNA levels. In addition to increasing the levels of GnRH receptors, estrogen can also sensitise gonadotrophs to the actions of GnRH by activating the PKC pathway [36]. It was found that after castration of male rats, their pituitary GnRH receptor concentration increased by 75 % at 24 hours and the GnRH receptor mRNA levels increased 5.2-fold after 21 days, while serum LH levels increased 10-fold. However, treatment with testosterone completely prevented the GnRH receptor and LH responses to castration. Ovariectomy of female rats also caused a 2- to 4-fold increase in serum LH levels in the first 3 days, with a second larger increase of 10- to 15-fold after 5 days. GnRH receptor concentration increased ± 2-fold on the third day after the operation and GnRH receptor mRNA levels increased 2-fold after 21 days. Treatment with estradiol alone, progesterone alone and a combination of progesterone and estradiol inhibited the increase in GnRH receptor concentration for up to 10 days [37, 38]. The other main female sex steroid, progesterone, had no stimulatory effect on GnRH receptor mRNA levels during the estrogen-induced LH surge and even exerted an inhibitory effect on GnRH receptor mRNA expression at the offset of the LH surge in adult rats [34].

Taken together these results indicate that the increased pituitary binding of GnRH is a significant component of the mechanism responsible for postcastration elevations of gonadotropin secretion and that the regulation of GnRH receptor number and upregulation of GnRH receptor mRNA expression at the time of preovulatory LH and FSH surges is probably due to actions of steroids exerted indirectly through increased GnRH neurosecretion. In fact Loumaye, E. and Catt, K..J. (1982) [39] observed an increase in the number of binding sites, i.e. number of GnRH receptors when they treated primary cultured pituitary cells with GnRH-agonist. Other evidence by Cho, B.N. *et al.* (1994) [40] showed that progesterone can stimulate GnRH gene expression in the hypothalamus of ovariectomised, estrogen treated adult rats. Chappell, P.E. *et al.* (2000) [41, 42] further elucidated the role of the sex steroids on the GnRH surges by showing that estrogen is in fact the main stimulant and that the progesterone receptor functions as a estrogen-induced factor whose activation is necessary for the stimulation of the GnRH surges and that cAMP plays a role in this stimulatory effect. This may seem in contrast to the findings quoted above that state that progesterone appears to have an inhibitory effect on GnRH receptor mRNA expression. However Chappell, P.E. and Levine, J.E. (2000) [41] proposed that progesterone receptor (PR) trans-activation may initially be stimulated by a neural signal and be independent of changes in levels of circulating progesterone. It would also seem likely that pulsatile GnRH neurosecretion contributes to the maintenance of basal GnRH receptor mRNA expression at times in the estrous cycle when circulation of gonadal steroids are low [34]. In conclusion, it seems that the stimulatory effect of estrogen and progesterone require the presence of a hypothalamic input in the form of increased pulsatile GnRH secretion.

The results observed in clonal cell lines are not so clear cut as those in whole animals. McArdle, C.A. *et al.* (1992) [31] found in αT3 cells that estradiol alone reduced GnRH receptor numbers. Furthermore, estradiol also reduced the efficiency of coupling of residual GnRH receptors to second messengers.

Experiments were also performed in another clonal cell line called LβT2 (gonadotropes), which express LHβ, α-subunit and GnRH receptor mRNA [43]. These cells respond to pulsatile GnRH administration with LH secretion and a significant increase in GnRH receptor mRNA, but estradiol alone or in combination with pulsatile GnRH had no effect on GnRH receptor mRNA levels. However a combination of estradiol and dexamethasone led to a dramatic increase of GnRH receptor mRNA. It was also found that the treatment of these cells with a combination of estradiol and dexamethasone plus pulsatile GnRH led to an even larger increase in GnRH receptor mRNA levels [43]. The results for GnRH receptor mRNA levels are consistent with those obtained in our laboratory, where it was found that estrogen, progesterone or testosterone alone had no effect on GnRH mRNA levels in αT3 cells [44]. In conclusion, it would seem that estrogen alone has no effect on the number and mRNA levels of GnRH receptors in clonal cell lines. However, when estrogen and pulsatile GnRH are combined, one sees an increase in GnRH receptor mRNA levels.

In whole animals, however, it appears that the effect of the sex steroids, estrogen progesterone and testosterone on the GnRH receptor gene are indirect, i.e. their main mechanism of action is to increase expression and secretion of certain genes in the hypothalamus. In clonal cell lines this hypothalamic input is absent and therefore estradiol alone is unable to increase GnRH receptor levels. As mentioned above, during the estrous phase, when circulating estrogen and progesterone levels are high, the increase in GnRH receptor mRNA and protein levels are due to an indirect effect; estrogen will feedback to the hypothalamus (see figure 1.4) and stimulate secretion of GnRH. This enhanced secretion of GnRH would then have a stimulatory effect on its own receptor levels.

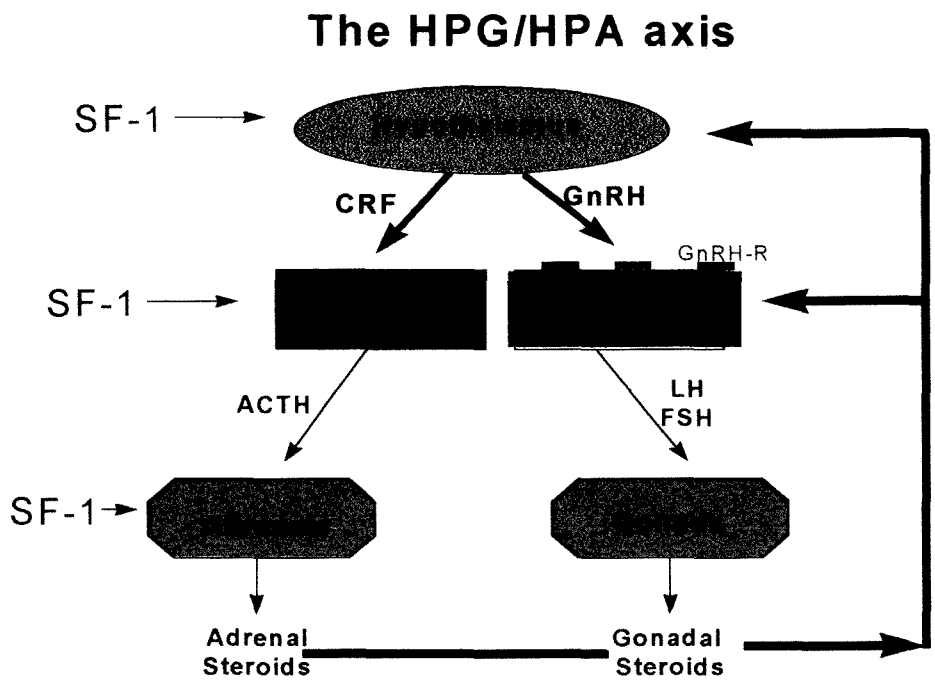


Figure 1.4: A schematic representation of the Hypothalamic-Pituitary-Gonadal/Adrenal axis (HPG and HPA respectively). The trophic peptide hormones GnRH and corticotropin-releasing factor (CRF) are released from the hypothalamus into the bloodstream. They bind to their specific receptors in the pituitary and results in the transcription and release of the gonadotropins LH and FSH, and in the case of CRF in adrenocorticotrophic hormone (ACTH). LH , FSH and ACTH then travel to their respective target organs, the gonads and adrenals, and result in the synthesis and release of the steroid hormones. These include estrogen, progesterone, testosterone and cortisol. The steroid hormones are able to feedback i.e. they can regulate genes higher up in the axis like the GnRH receptor gene. Steroidogenic factor-1 (SF-1) is expressed in the hypothalamus, pituitary, adrenals and gonads and regulate genes involved in the above processes (see sections 3,4 and 5). Figure compiled by G. Styger.

D.) Regulation of the GnRH receptor by GnRH

Thus it would seem that in the regulation of the GnRH receptor gene, its own ligand, GnRH, plays the most important role. It was shown that in rat pituitary primary cultures, GnRH receptor is up-regulated by GnRH pulses [12]. Pulsatile GnRH was also found to up-regulate GnRH receptors levels in adult female rat cultured pituitary cells [39]. Tsutsumi, M. *et al.* (1993) [45] performed GnRH binding studies and found that after exposure of αT3 cells

to 10^{-10} or 10^{-8} M GnRH for 20 minutes there was a 50% increase in receptor number. However despite the increase in receptor number, cytosolic and total GnRH receptor mRNA levels remained unaltered, but the ability of the mRNA to direct synthesis of new receptor, was increased about 2-fold. They concluded that the upregulation of its own receptor by GnRH involves, in part, modulation of the capacity of cellular mRNA to direct the biosynthesis of GnRH receptor.

The importance of GnRH pulse amplitude and frequency on GnRH receptor mRNA levels was investigated in gonadectomised rats with hormone replacement [46]. In males, GnRH receptor mRNA levels were increased by 3-fold at all pulse levels (5-25 ng/pulse) but only low doses were found to be effective in females with a 2-fold increase in mRNA levels. Female rats were more sensitive to changes in the frequency of the GnRH pulse than males. When pulsatile GnRH was combined with estradiol (in females) the effect was a synergistic one, once again proving that estrogen can play a role in the GnRH-stimulated GnRH receptor increase. It was also found that these increases involve both protein as well as the mRNA levels of the GnRH receptor in cultured pituitary cells and in whole animals [17]. The results also suggested that the upregulation was not just a transcriptional effect but also involves the increase in mRNA activity i.e. a translational effect.

By contrast, continuous treatment of αT3 cells with GnRH or its agonist reduced GnRH receptor numbers in a time- and dose-dependent manner and led to a 80% reduction in receptor protein levels. A decrease in GnRH receptor mRNA levels was also observed, but this was consistently less than the decline in receptor number [12, 47]. Tsutsumi, M. *et al.* (1995) [48] found that after exposure to 1 μM GnRH for 24 hours the number of GnRH receptor binding sites in αT3 cells decreased to 25% of the control. No corresponding changes were seen in GnRH receptor mRNA levels, but the ability of cellular mRNA isolated from αT3 cells to direct the synthesis of functional GnRH receptor was decreased. The cellular basis for the decrease in GnRH

receptor levels after continuous GnRH treatment could thus be explained by a combination of the following mechanisms: down-regulation of GnRH receptor gene transcription, as well as decreased GnRH receptor mRNA translation [12, 47, 48].

It is clear from the above that pulsatile GnRH treatment causes an increase in GnRH receptor protein and mRNA levels, whilst continuous GnRH causes a decrease in both protein and mRNA levels of the GnRH receptor. The interesting question remains however: What is the mechanism by which GnRH regulates the expression of its own receptor? In other words what are the second messenger pathways responsible for relaying the pulsatile GnRH signal? The first candidate would be the PKC pathway, involving a influx of Ca^{2+} into the cells. Cultured female rat pituitary cells in perfusion given 180-min pulses of the Ca^{2+} channel activator BayK 8644 showed a 41% increase in GnRH receptor mRNA levels, showing that this could be an important step in the transmission of GnRH pulse signals from the membrane to different genes including the GnRH receptor gene [17]. Alarid, E.T. *et al.* (1995) [49] showed no effect with GnRH and TPA, a phorbol ester and PKC activator, on GnRH receptor mRNA levels, in αT3 cells, but showed that forskolin decreased GnRH receptor mRNA levels in a time dependent manner, by approximately 6-fold in αT3 cells. Forskolin was found, however, not to exert its effect at the transcriptional level, but rather to decrease mRNA stability. These results, however, are in direct contrast with those of Lin, X. and Conn, P.M. (1998, 1999) [28, 29] who found that GnRH activates GnRH receptor transcriptional activity through multiple signal transduction pathways i.e. the PKC and Ca^{2+} pathways and the cAMP-PKA pathway. However, their experiments were done in GGH_3 cells, where the GnRH receptor might be associated with a different G protein than in αT3 cells, leading to the activation of different second messenger pathways. A recent study in GGH_3 cells by Stanislaus, D. *et al.* (1998) [11] showed that the GnRH receptor could associate with $G_i\alpha$, $G_s\alpha$ and $G_{q/11}\alpha$ proteins in a single cell, suggesting that the GnRH-stimulated increase in GnRH receptor levels could occur via the

differential use of the different signal transduction pathways. The role of the PKA and PKC pathways on GnRH receptor gene regulation is discussed in more detail in section 3D.

2. Structure of the GnRH receptor gene and promoter region

The structures of the mouse [50, 51], rat [5, 52], sheep [53] and human [54, 55] GnRH receptor genes are known and have been cloned. These genes have a high degree of sequence homology in the coding region, with the seven transmembrane domains spaced over three exons. However, they differ dramatically in the sizes of the two introns as well as the sequence and length of the 5'-untranslated regions (5'-UTR), i.e. regions that are transcribed, but not translated, as well as the 5'-flanking region i.e. sequences that are neither transcribed nor translated. The lack of homology in the 5'-flanking region implies that the GnRH receptor gene could be regulated differently in each different species. However, many putative *cis*-elements are conserved (see figure 1.5), indicating some conservation of important functional elements. The structures of the GnRH receptor genes from mouse, rat and human will now be described in more detail, highlighting the different confirmed *cis*-elements, established in functional studies, as well as some putative *cis*-elements inferred from consensus sequences.

It has been found that the 1.2 kb of the 5'-flanking region of mouse GnRH receptor gene contains regulatory sequences that direct cell-specific and GnRH responsive gene expression to the pituitary *in vivo*. Albarracin, C.T. *et al.* (1999) [56] developed a stable transgenic mouse model by linking the 5'-flanking region of the mouse GnRH receptor to the simian 40 virus T antigen, and these mice consistently developed tumours in the pituitary. Other researchers created a transgenic mouse line that contained 1900 bp of 5'-flanking region from the mouse GnRH receptor gene linked to a luciferase gene [57]. This 1900 bp was sufficient to direct tissue-specific expression, i.e.

in pituitary, brain and testis, in the transgenic mice. Luciferase expression was abrogated after GnRH immunoneutralisation suggesting that one or more elements conferring GnRH responsiveness are located in this 1900 bp mGnRH receptor of 5'-flanking region.

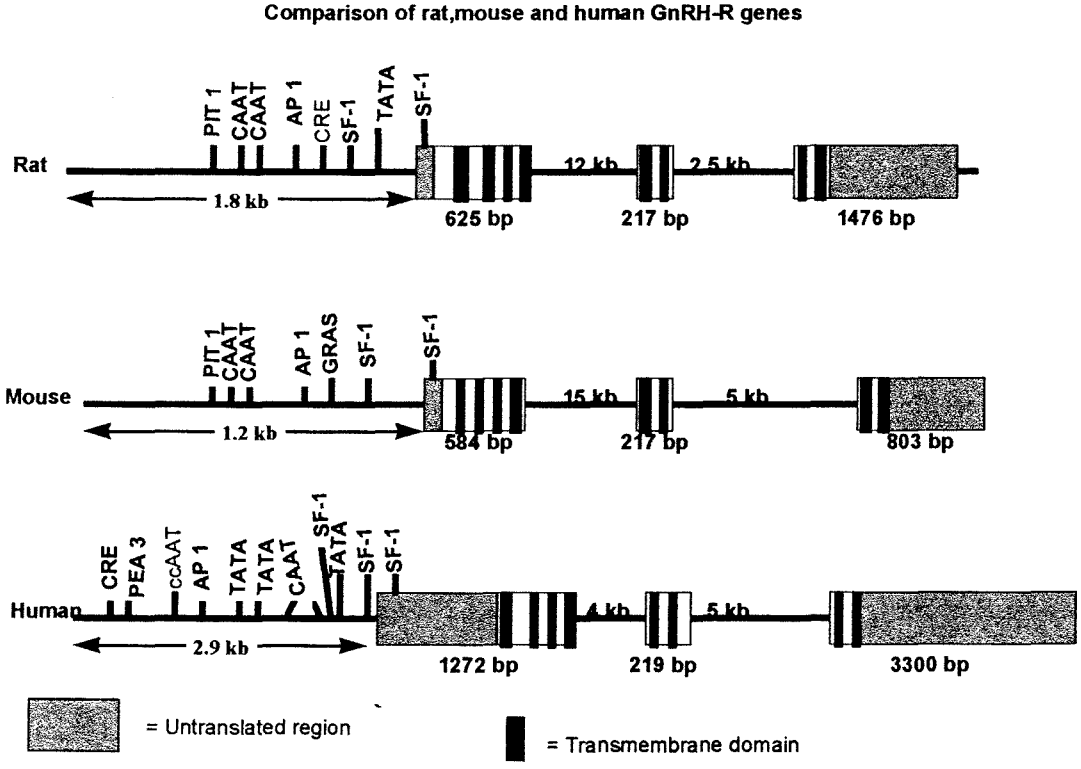


Figure 1.5: Comparison of the rat, mouse and human GnRH receptor genes, showing the 5'-flanking or promoter region as well as the three exons and two introns. The figure is not drawn to scale and is merely given to demonstrate the differences between promoter regions of the three different species. The relative sizes for the promoter regions are given underneath each example. The abbreviations are as follows: Pit-1, Pituitary specific response element 1; CAAT, CAAT box binding element; AP-1, Activator Protein 1 response element; CRE, cAMP responsive element; SF-1, Steroidogenic Factor 1 recognition site for the mouse promoter (note that the SF-1 site in the 5'-untranslated region or 5'UTR was previously known as the Gonadotrope Specific Element (GSE)); TATA, TATA box binding element; GRAS, GnRH receptor activating sequence and PEA-3, phorbol ester sensitive sequence. Compiled by G. Styger based on [51].

The mouse GnRH receptor (mGnRH-R) gene contains three exons with sizes from 217 bp to 803 bp (see figure 1.5). These are separated by two introns, with sizes of 15 kb for intron I and 5 kb for intron II [50]. The major transcriptional start site is situated at position -63 relative to the ATG translational start site, while two minor transcription start sites were found at -70 and -106 relative to the ATG codon [58]. Interestingly, no TATA boxes were found upstream, but it is well known that G-protein coupled receptor genes often lack such *cis*-elements. Alternatively, a TCTAA motif and a GATA motif at -30 in the mGnRH-R gene may fulfil the role of the TATA box. However Clay, C.M. *et al.* (1995) [59] found a second major transcription start site 200 bp (i.e. at -200) upstream of the translation initiation codon, which may be associated with a TATA box.

The positions of some putative and established *cis*-elements are shown in figure 1.5. One of the *cis*-elements identified by sequence homology includes a binding site for activating protein-1 (AP-1). This has been shown to confer protein kinase C (PKC) sensitivity to the promoter [60, 61]. Recently another important regulatory element called the GnRH receptor activating sequence (GRAS) was identified [62]. Currently the protein that binds to this site is still unknown. There is also no known function for the CAAT element. The ubiquitous pituitary-specific (Pit 1) response element was also identified by sequence homology in the 5'-flanking region. Another interesting element is the gonadotrope-specific element or (GSE). This element is the same as the SF-1 site located in the 5'-untranslated region (5'-UTR) and was first thought to play a role in conferring tissue specificity to this gene. The GSE was first identified in, and thought to be responsible for tissue specific expression of the α-subunit [7] and LHβ genes [63, 64, 65]. However, no such site was found in the FSHβ gene [66]. The 54-kilodalton protein that binds to the GSE element in these gonadotropin genes was recently identified as steroidogenic factor-1 (SF-1) [6, 7]. There are two putative SF-1 binding sites in the mouse GnRH receptor gene promoter, one at position -247 relative to the ATG start codon and another one at position -14 [58]. The latter site is downstream of

the transcription start site and therefore situated in the 5'-UTR. Interestingly there are no consensus cAMP response elements in the promoter region [50, 51]. The *cis*-elements and transcription factors shown to be involved in regulating the mouse GnRH-R gene will be discussed in greater detail in section 3.

The rat GnRH receptor (rGnRH-R) gene also consists of three exons with sizes of 625, 217 and 1476 nucleotides respectively. The first intron is at least 12 kb whilst the second intron is only 2.5 kb. The transcriptions start site is 103 nucleotides upstream of the ATG codon and there is a TATA box near to it. Sequence analysis data shows that the 1.8 kb 5'-flanking region contains two SF-1 binding sites, once again one upstream of the transcription start site and another one downstream in the 5'-UTR, just like in the mouse promoter (see figure 1.5). There is also an AP-1 site, CAAT boxes, a Pit-1 binding site and a potential cAMP response element (CRE)-like sequence. The 1.5 kb 5'-flanking region was linked to a luciferase reporter gene and could be expressed in pituitary αT3 and hypothalamic GT1-7, but not in kidney COS-7 cells, showing that elements conferring tissue-, but not cell-specificity were present in this region [52] in αT3 cells. Forskolin responsiveness was also present in this region and thought to be mediated via the putative CRE. However, phorbol esters had no effect, in αT3 cells, which is surprising considering the presence of a functional AP-1 site [52].

The human GnRH receptor (hGnRH-R) gene has a 5'-UTR of 1272 bp (see figure 1.5). There are seven TATA and six CAAT sequences in the promoter region as well as 18 transcriptional start sites [55]. A 1.2 kb of the 5'-flanking region linked to a luciferase reporter gene could be expressed in human endometrial tumour cell line (HEC-1A) and human breast tumour (MCF-7), but not in a mouse αT3 gonadotrope cell line [54]. This inability of the promoter to drive transcription in the mouse system, suggests the possibility that this gene is differentially regulated in different species. Several putative regulatory sequences have also been found on the basis of sequence

analysis and include sites for PEA-3, a site which is sensitive to phorbol esters; AP-1 and AP-2, which confer PKC responsiveness, and the pituitary specific transcription factor (Pit-1). There are also additional putative recognition sites for the glucorticoid/progesterone- and thyroid hormone receptors as well as the cyclic adenosine 3'-5' monophosphate (cAMP) response element binding protein (CREB) [54].

3. Factors interacting with *cis*-elements in the GnRH receptor promoter

A.) Elements involved in basal and cell-specific expression of the GnRH receptor gene.

Since most work on regulation of GnRH receptor expression has been done on the mouse GnRH receptor gene promoter, also the subject of the present study, most of the evidence presented shall pertain to the mouse model. As seen above, the promoters of the GnRH receptor genes from different species, differ in the number and positioning of conserved putative elements, while some have unique elements not found in other species. One area where conservation would be probable would be in the elements that confer cell- and tissue-specificity to the gene. It has previously been shown that ±500 bp of 5'-flanking region of mouse GnRH receptor promoter can bestow cell-specific expression in αT3 cells and elements responsible for this must reside in this area [58, 59, 62]. One likely candidate for cell-specific expression is the gonadotrope-specific element or GSE. In fact the GSE has been shown to confer pituitary-specific expression to the α-subunit gene in αT3 cells in conjunction with a pituitary glycoprotein basal element [63]. The protein that binds to the GSE *in vitro* was later identified as SF-1 [6, 7] and such sites were also found in the promoter regions of the GnRH receptor [62] and LHβ-subunit genes [63, 64, 65]. The above evidence would point to a possible role for SF-1 in tissue- or cell-specific expression of the GnRH receptor gene. However Clay, C.M. *et al.* (1995) [59] and work previously

done in our laboratory [58] showed that the putative SF-1 site 1, located immediately upstream of the ATG start codon (see figure 1.6), is not involved in the regulation of cell-specific activity of the GnRH receptor, but that it might still play a role in tissue-specificity. Clay and his colleagues suggested that elements residing between -500 and -200 fulfil the function of cell-specificity. This area includes the second SF-1-like site (site 2) located at -247 upstream of the ATG start codon. Work done by Duval, D.L. *et al.* (1997) [62] showed that basal activity of the GnRH receptor gene requires elements residing at -350 to -235. They also found that mutation of this putative SF-1-like site 2 caused a 58% drop in promoter activity.

More recent work by this group [67] identified a tripartite enhancer for the GnRH receptor gene consisting of the above mentioned SF-1-like site 2, an Activator protein (AP-1) site, located at -337 to -331, and a novel GnRH Receptor Activating Sequence (GRAS), located at -392 to -381, with the following sequence CTAGTCACAACA (see figure 1.6). Mutation of any one element alone reduced promoter activity by 60%, while simultaneous mutation of any two elements reduced promoter activity by 80% and with the simultaneous mutation of all three elements there was no discernible promoter activity. It was also found that 3 copies of the GRAS element linked to a reporter gene were sufficient for cell-specific activity. They thus proposed that the full basal activity of the GnRH receptor gene promoter requires the presence of all three elements and that GRAS plays the major role in cell-specific activity. It is clear then that the SF-1-like site 2 plays an important role in the regulation of the basal and cell-specific regulation of the GnRH receptor, but that it acts in concert with the AP-1- and GRAS elements. The factor that binds to the GRAS element has not yet been identified. Interestingly, the GRAS element was recently found by the same group to mediate the response to activin and inhibin [34]. Activin and inhibin are members of the TGF-β family of growth factors, and have been shown to regulate the levels of GnRH receptor protein and to increase transcription of the GnRH receptor gene, although the mechanism is not clear. The authors

showed that 600 bp of the 5'-flanking region of the mouse GnRH receptor was sufficient to confer activin responsiveness in αT3 cells. They also showed that mutation of the GRAS element inhibited the ability to respond to activin.

The basal and cell-specific regulation of the human GnRH receptor (hGnRH-R) differs slightly from the mouse GnRH receptor gene. None of the three SF-1-like sites in the hGnRH-R are in close proximity to an AP-1 or GRAS element. It was found that a SF-1-like site situated at -134 relative to the ATG start site in the human GnRH receptor promoter was sufficient to mediate cell specific expression of this gene [68].

Taken together, all the above results show that the basal and cell-specific expression of the GnRH receptor gene is dependent on a tripartite enhancer consisting of a DNA-binding site for SF-1 (site 2), an AP-1 binding site and a GRAS element that is responsive to the actions of activin and inhibin. However once again the mechanism by which this occurs has not yet been elucidated. Of the three elements it would also seem that the GRAS element has the major role to play in cell-specificity.

B.) Elements involved in steroid-dependent expression of the GnRH receptor gene.

The above scenario represents only the case of basal or unstimulated expression of the GnRH receptor gene. However as seen in the first section the GnRH receptor gene may be under control from various steroids as well as its ligand GnRH. From section 1 C it can be seen that the mechanism whereby steroids regulate the GnRH receptor gene is not yet clear. One can thus not say that the effects on GnRH receptor mRNA levels by steroids such as estradiol are due to a transcriptional effect. These changes in mRNA levels can merely be due to differences in mRNA stability. If these effects are

at the transcriptional level, they would be mediated by transcription factors binding to specific *cis*-elements in the promoter region of the gene. However no functional *cis*-elements for testosterone (T) or progesterone (P) have been identified for the GnRH receptor promoter for any species. However, Duval, D.L. *et al.* (2000) [32] recently showed that 9100 bp of the 5'-flanking region of the ovine GnRH receptor gene was sensitive to treatment with GnRH-agonist alone and estrogen alone in transgenic mice, but not in αT3 cells. A 2700 bp GnRH receptor promoter transgenic mouse line was also responsive to these compounds. Thus it does appear that the mouse GnRH-R promoter contains an as yet unidentified estrogen responsive element within 2700 bp of the 5'-flank. Although the final picture in the transcriptional regulation of the GnRH receptor gene by estrogen is still unclear these transgenic mice could be very useful in further studies.

C.) Elements involved in GnRH-dependent expression of the GnRH receptor gene.

As described in section 1 D it appears that GnRH regulates mRNA levels and synthesis of its own receptor. In section 2, transgenic mouse experiments have also shown that the mouse GnRH receptor promoter is responsive to GnRH [57]. Consistent with these results in animals, Lin, X. & Conn, P.M. (1998) [28] found that in GGH_3 cells, GnRH or GnRH-agonist stimulated reporter activity in a dose-dependant manner. Treatment with cAMP stimulated reporter activity and pre-treatment of cells with cAMP enhanced the effect of the GnRH-agonist. A very interesting finding was that treatment with GnRH-agonist significantly stimulated cAMP release in these cells. They thus postulated that both GnRH and cAMP activate GnRH receptor transcriptional activity and that part of the GnRH activation is mediated via the cAMP pathway.

They further found that the response elements for GnRH and cAMP reside at two different sites: between -394 and -318 and between -318 and -1 respectively, relative to the ATG start codon. Their findings are consistent with the reports above and place the cAMP response element in the region that contains both SF-1-like sites (-245 to -237 and -15 to -8), and the GnRH responsive element in the region that contains the AP-1 site at -337 to -331 and a GRAS site at -392 to -381. They suggested that the transcriptional factors acting via these sites could be involved in mediating the effects of GnRH and cAMP. Maya-Nunez, G. & Conn, P.M. (1999) [69], also working in GGH_3 cells, reported that the mouse GnRH receptor gene contains a CRE-like sequence at -107 to -100 similar to one in the protooncogene c-*fos* sequence TGACGTTT (see figure 1.6). This putative CRE contains only the 5'-half consensus palindromic sequence and the protein that bound to this site has not yet been identified. Deletion within this element caused a complete loss in basal and GnRH-agonist- and forskolin-stimulated transcription. Maya-Nunez, G. & Conn, P.M. (1999) [69] also identified the presence of a putative repressor element at -343 to -335 (see figure 1.6). Deletion of this element increased GnRH receptor promoter activity in basal and GnRH-agonist-, phorbol ester-, and forskolin-stimulated GGH_3 cells. From the above evidence it appeared as if elements in the region -392 to -237 relative to the ATG start codon, plus a newly discovered CRE-like element as well as a repressor element all played a role in mediating the GnRH response on the mGnRH receptor gene.

Evidence for the importance of the tripartite enhancer region in response to a GnRH signal was also found in the αT3 cell line by White, B.R. *et al.* (1999) [60] who mutated each component of the tripartite enhancer and found that GnRH responsiveness was lost only when the AP-1 site was mutated. Norwitz, E.R. *et al.* (1999) [70], also using the αT3 cell line, localised the GnRH responsive elements to two distinct areas and showed that both seem necessary and sufficient to mediate a full GnRH response. The first element,

with the sequence 5'-TATGAGTC-3', was named the Sequence Underlying Responsiveness to GnRH-1 element or SURG-1 and is situated at position -339 to -332 and overlaps the consensus AP-1 binding site. The second element SURG-2, with sequence 5'-GCTAATTG-3', lies at -355 to -348 and appears to be novel (see figure 1.6). Interestingly, studies done on the human GnRH receptor gene in αT3 cells [61] showed that the transcriptional down regulation of the hGnRH-R gene by GnRH is mediated via two putative AP-1 sites in the promoter region. They also show that the activation of the PKC pathway is important in controlling hGnRH-R gene expression in αT3 cells.

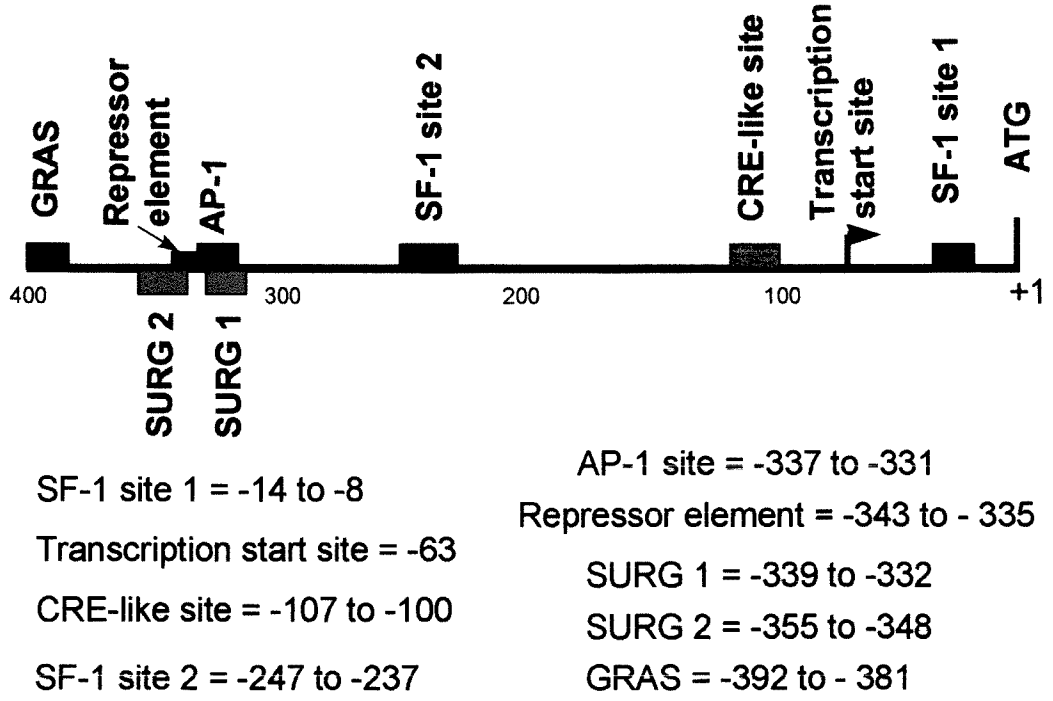


Figure 1.6: Schematic representation of 400 basepairs of the mouse GnRH receptor gene promoter. The scheme is drawn to scale and shows the positioning of the major *cis*-elements involved in basal- and GnRH-dependent expression of the mGnRH-R gene. All elements are numbered relative to the ATG start codon. Underneath the scheme the actual positions of the elements are indicated, i.e. the ATG or translational start site at position +1 [50], the SF-1 like site 1 at position -14 to -8 [50], the major transcriptional start site at position -63 [58], the putative CRE-like sequence at position -107 to -100 [69], the second SF-1 site at position -247 to -237 [67], the AP-1 site at -337 to - 331 [67] and the GRAS element at position -392 to -381 [67]. Also indicated is the repressor element found in position -343 to -335 [69], as well as the two SURG elements found at positions -339 to -332 (no. 1) and -355 to -348 (no. 2) [70]. Figure compiled by G. Styger from information in above references.

The summary of all the above results would be that GnRH-dependent transcriptional regulation of the mGnRH receptor gene involves several elements in the promoter region of this gene. From the work by Lin, X. & Conn, P.M. (1998) [28] it would seem that the region containing the tripartite enhancer plays a role in this regard. Evidence was also given that the PKA pathway is involved via a putative CRE-like element [69]. All of the above experiments were however performed in the GGH_3 cell line. Other experiments done in αT3 cells [60, 70] showed that the AP-1 site, together with the SURG-1 and SURG-2 elements are the important elements in the mediation of the GnRH response. The issue of which second messenger pathway is used to mediate the GnRH response will be reviewed in more detail in the next section.

D.) The roles of the PKA and PKC/Ca^{2+} signal transduction pathways in GnRH-dependent regulation of the GnRH receptor gene

The roles that the major signal transduction pathways i.e. PKC/Ca^{2+} and PKA play in the regulation of GnRH receptor gene transcription is a very complicated issue. Lin, X. & Conn, P.M. (1998) [28] and Maya-Nunez, G. & Conn, P.M. (1999) [69] found that the major second messenger pathway involved in GnRH-dependent GnRH receptor gene regulation is the PKA pathway. It is nonetheless important to remember that both of the above groups make use of the GGH_3 cell line. These cells are not natural gonadotropes, but rather somatolactotropes stably transfected with the GnRH receptor gene. They express the GnRH receptor on their cell surfaces, but may well lack other internal factors that play a role in gonadotrope specific expression like SF-1 or other nuclear receptors. Evidence pointing to the importance of the PKC pathway was found by Norwitz, E.R. *et al.* (1999) [70] and Cheng, K.W. *et al.* (2000) [61] and their data suggest that the response of the mouse and the human GnRH receptor gene to GnRH is mediated via the PKC and not the PKA. pathway. These authors made use of the αT3 cell

line in their experiments and the seemingly contrary evidence concerning the PKA *vs.* PKC pathways could possibly be explained by the differences between the two cell lines (GGH_3 *vs* αT3).

Perhaps the most plausible scenario is the one suggested by Lin, X. and Conn, P.M. (1999) [29] who propose that GnRH activates transcription of the GnRH receptor gene through both the PKA and PKC and Ca^{2+} pathways in GGH_3 cells. Some of the evidence they present is that the activation of PKC significantly stimulated reporter activity, whilst inhibition of PKC or PKC depletion blocked PKC- or GnRH-agonist stimulation of the GnRH receptor promoter. Also, GnRH-agonist stimulated reporter activity was inhibited by preventing the external Ca^{2+} influx with EGTA, or Ca^{2+} ion channel antagonist D600. They also find that the MAPK pathway is involved in repression of basal activity and GnRH-agonist stimulated GnRH receptor promoter activity. The mechanism of repression of basal activity is unknown but it might be due to phosphorylation of a repressor transcription factor. White, B.R. *et al.* (1999) [60] and Norwitz, E.R. *et al.* (1999) [70] found that ERK kinases partly mediate the GnRH regulation of the GnRH receptor gene in αT3 cells. Furthermore, members of the Fos/Jun heterodimer superfamily are part of the protein complex formed on SURG-2 and these proteins are induced by GnRH stimulation.

As can be seen the regulation of transcription of the GnRH receptor gene is a very complex situation where multiple factors and *cis*- and *trans*-elements play a part. In summary it can be said that the basal, unstimulated expression of the GnRH receptor gene involves a tripartite basal enhancer consisting of an AP-1, SF-1-like (site 2) and GRAS element. The SF-like site 1 seems to be unimportant in basal expression, however some constructs used may not have contained a complete site 1 (see conclusions). It is still unclear which of these three elements confer tissue- or cell specificity, but the GRAS element seems to play the major role in this regard. The AP-1 site and nearby novel elements SURG-1 and SURG-2 are the prime candidates for conferring

GnRH responsiveness to the gene, as mutation of these sites cause the GnRH receptor gene to be insensitive to GnRH. Lastly the second messenger pathway involved in the GnRH signal transduction appears to be the PKA pathway in GGH_3 cells, while the PKC/Ca^{2+} pathway takes precedence in αT3 cells. This has however not been clearly established and the possibility that the GnRH signal involves the differential use of both pathways remains. Whatever the case may be, the PKA response element identified as a putative CRE site is likely to play a role. However, this site has yet to be proven to be functionally recognised by any factor. Even if the GnRH-dependent regulation of GnRH receptor gene transcription is mediated solely by PKC in αT3 cells, the possibility exists that the gene is regulated by the PKA pathway via other signalling molecules. The PKC response element was clearly identified as the AP-1 and SURG-1 and SURG-2 elements. The presence of a repressor element was also investigated, but whether this element has a physiological role to play remains to be seen. Of course the regulation of a single gene cannot be looked at in isolation and will depend on a variety of other factors. For example the manner by which regulation of the GnRH receptor gene is achieved, is likely to be dependent on other external factors such as developmental stage, steroid milieu, stress condition, health condition and a myriad of other factors. It is however clear that SF-1 protein plays a pivotal role, together with various other factors, in the transcriptional regulation of the GnRH receptor. In the next section the role of SF-1 in gene regulation will be reviewed in more detail.

4. The role of SF-1 in gene regulation

Steroidogenic factor 1 (SF-1) was first discovered as a key regulator of steroidogenic enzyme gene expression and was also called adrenal 4-binding protein or Ad4BP, because it was identified as the protein that bound to the Ad4 *cis*-element of the bovine cytochrome P-450(11β) gene (CYP11B) [71]. The protein was called steroidogenic factor because it was first found only in

steroidogenic cells and thought to play an important part in the cell-specific expression of the cytochrome P-450 steroid hydroxylase genes. SF-1 protein was purified and it's cDNA isolated by two independent groups; Lala, D.S. *et al.* (1992) [72] from the mouse and by Honda, S. *et al.* (1993) [71] from bovine tissue. They both identified a 53 kilodalton protein and a 1383 bp cDNA that encoded for a protein of 461 amino acids.

From the cDNA sequence it was discovered that SF-1 was a member of the nuclear receptor superfamily, the members of which include the glucocorticoid, mineralcorticoid, progesterone, androgen, estrogen, thyroid hormone, and retinoid X receptors [73]. These proteins have conserved functional domains that include two zinc fingers, a proline rich domain (proposed to mediate transcriptional activation), a potential site for phosphorylation by cAMP-dependent protein kinase and an AF-2 transactivation domain [74]. SF-1 is a member of the family of orphan nuclear receptors. These are receptors that have no identified ligand and are possibly regulated by other means, and also include the transcription factors COUP-TF and NUR-77 [7]. Another surprise was that the cDNA showed homology to the mammalian homologue of the *Drosophilia* transcription factor *fushi tarazu*-factor 1 or FTZ-F1 that regulates the *fushi tarazu* gene, as well as embryonal long terminal repeat-binding protein (ELP) which is expressed in mouse embryonal carcinoma cells [71, 72, 74]. Characterisation of the mouse *FTZ-F1* gene by Ikeda, Y. *et al.* (1993) [75] showed that both SF-1 and ELP are encoded by the same gene and that there are shared exons encoding common regions and alternative promoter and 3'-exons which give rise to the differences between the two transcripts.

To further understand the role of SF-1 in steroidogenesis the spatial and temporal developmental profile of SF-1 expression was studied. Ikeda, Y. *et al.* (1994) [76] found SF-1 transcripts in the urogenital ridge, the precursor of the gonads, adrenal glands and kidneys, at embryonic day 9 (E9). By E11 when the adrenals and gonads are clearly separate, SF-1 was present in the adrenal primordium and the genital ridge. By E12.5 to E13.5, SF-1 transcripts

were localised in the outer cortical region of the adrenal gland [74, 77] and after sexual differentiation, about E14, SF-1 was significantly expressed in the Leydig and Sertoli cells of the testis, but there was almost no expression in the ovaries [78]. SF-1 was also found in the embryonic forebrain, later to become the hypothalamus and pituitary, suggesting that it plays a more global role in the hypothalamic-pituitary-gonadal/adrenal axis than simply regulating steroidogenesis.

Evidence for the above statement was obtained when the role of SF-1 *in vivo* was investigated using *FTZ-F1* knockout mice. Luo, X. *et al.* (1994) [79] created chimerical animals by targeted disruption of the *Ftz-F1* gene. They show that -/- knockout animals were born at the expected frequency of 25%, indicating that SF-1 is not required for embryonic survival [77]. However all the -/- animals had external female genitalia, irrespective of genetic sex, a failure to produce testicular androgens, [74, 77] and died shortly after birth due to corticosteroid deficiency, indicating the important role that SF-1 plays in androgen and corticosteroid biosynthesis. A very interesting observation about these -/- animals was that their adrenal glands and gonads were completely absent, once again revealing the key role that SF-1 plays, not only in steroidogenesis, but also in the development of these tissues [74, 77, 79].

As SF-1 was also found to be expressed in the embryonic forebrain and later in the hypothalamus and anterior pituitary, it suggested that further abnormalities would be likely to occur in these animals. Ingraham, H.A. *et al.* (1994) [6] found that the pituitaries of the knockout mice were devoid of LH, FSH and GnRH receptor protein and mRNA which are all classical markers for gonadotropes. Indeed Asa, S.L. *et al.* (1996) [80] showed that SF-1 is preferentially expressed in gonadotrope cells of the pituitary. These animals also lacked the ventromedial hypothalamic nucleus, a region that contains high concentrations of steroid hormone receptors and has been implicated in female reproductive behaviour [77]. In another study the spleen of knockout mice showed severe developmental defects, i.e. impaired splenic vascular

architecture [81]. From these results it became apparent that SF-1 plays an important role in steroidal and gonadal development and regulation, and is not only involved the transcriptional regulation of the steroidogenic enzyme genes.

One explanation for the absence of LH, FSH and GnRH receptor protein and mRNA in the pituitary of SF-1 knockout mice can be gleaned from promoter analysis of these genes. Experimental analysis showed that SF-1 protein interacts with certain elements on the gene promoter regions of the α-subunit of the gonadotropin hormones [6, 7] and the LHβ subunit [63, 64, 65] as well as the GnRH receptor [62, 68] and regulates their expression. However it was found that treatment of the SF-1 knockout mice with GnRH restored the pituitary expression of LH and FSH, [74] suggesting that SF-1 is not essential for gonadotropin and GnRH receptor expression. This however raised the possibility that SF-1 acts not only on the gonadal/adrenal and pituitary levels of the HPG/HPA axis, but also at the hypothalamic level. The absence of SF-1 in these knockout mice did not influence the normal numbers and location of GnRH neurons [82], but might interfere with GnRH expression, secretion or delivery to the pituitary. This disruption in GnRH together with the fact that SF-1 plays a direct role in transcription of the gonadotropin and GnRH receptor genes, may explain why treatment with GnRH can only partially restore LH and FSH expression. This still does not explain why one can partially restore LH and FSH expression, when SF-1 is absent, since SF-1 is supposed to be very important for tissue-specific expression of these genes. One possible explanation might be that SF-1 can be substituted for by other orphan nuclear receptor family members.

It is thus well established that SF-1 is a key regulator at all levels of the HPG/HPA axis and that the absence of SF-1 results in serious developmental defects and the lack of expression of important genes. The question of which genes SF-1 regulates and the mechanism thereof will now be examined in more detail.

5. Gene regulation by SF-1

A.) The genes regulated by SF-1

Since SF-1 was first discovered as a regulator of the steroidogenic enzyme genes, most studies pertaining to SF-1 binding to DNA have been performed on these genes. It is well established that most members of the nuclear receptor superfamily bind to DNA in one of two ways, i.e. by direct or inverted dimerization. This means that the binding site consists of either directly repeated or inversely repeated half-sites, allowing the binding of two receptors next to each other. However SF-1 and its close relative Nur-77, bind to DNA as monomers, resulting in a third paradigm of nuclear receptor-DNA interaction [83].

SF-1 DNA-binding sites have been identified and shown to be functional in several promoters of genes involved in steroidogenesis (see figure 1.7) , i.e.: the cytochrome P450 aromatase gene [84, 85, 86, 87, 88], the cytochrome P450 side-chain cleavage gene [89, 90, 91], the gene encoding cytochrome P450 steroid 17α-hydroxylase/c17-20 lyase [92, 93, 94] and the steroidogenic acute regulatory (StAR) protein gene [95, 96, 97, 98] as well as the high density lipoprotein receptor, which mediates the uptake of cholesterol and cholesteryl esters, the substrates for steroidogenesis [99]. For most of these promoters, there is strong evidence that SF-1 protein is the transcription factor which mediates the response via the SF-1 *cis*-element.

As mentioned in section 4, SF-1 protein was later found to not just regulate the steroidogenic enzyme genes, but a variety of other genes as well. One of the effects of SF-1 knockout mice was that all animals had external female genitalia, suggesting that SF-1 protein played a role in the sex determination cascade that takes place shortly after testis development. A very important link in this cascade is Müllerian inhibiting substance or MIS. MIS is secreted by the Sertoli cells of the testis, causing cell-death of the Müllerian duct and

development of external male genitalia. SF-1 protein was found to bind to elements in the promoter region of the MIS gene [100, 101]. SF-1 protein was also found to be essential for basal transcription of the human ACTH receptor gene [102] and the oxytocin gene in the bovine ovary [103], as well as the gonadotropin α-subunit and LHβ-subunit and GnRH receptor genes in the pituitary gonadotropes, mentioned in section 3A. Additionally SF-1 *cis*-elements have been found to be positive regulators of rat granulosa cell differentiation and negative regulators of mitosis [104].

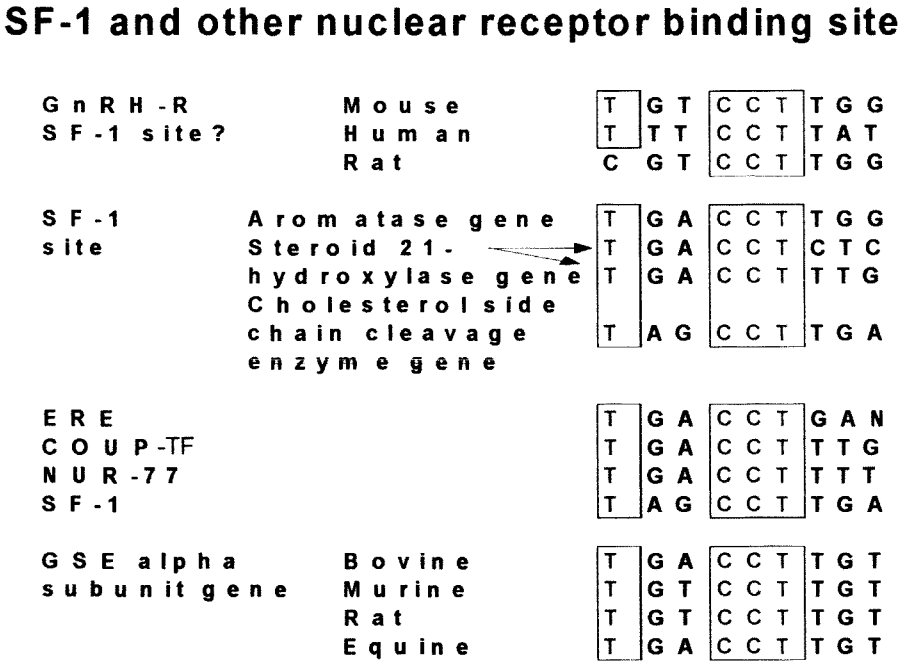


Figure 1.7: The DNA-binding sites for SF-1 and other nuclear receptors. The consensus DNA-binding sites for SF-1 are shown for various genes, including the GnRH receptor [50], aromatase [84, 85, 86, 87, 88], steroid 21-hydroxylase [92, 93, 94], cholesterol side-chain cleavage [89, 90, 91] and α-subunit genes [6, 7]. The SF-1 site 1 is shown for the mouse gene, the sequence of site 2 is TGGCCTTCA. For the rat, SF-1 site 1 is shown, the sequence of site 2 is TGGCCTTCA [52]. The human SF-1 site 1 is also shown, the sequence for site 2 is TATCCTTAA and for site 3, TTTCCTTGA [54]. The DNA-binding sites for other nuclear receptors i.e. ERE, COUP-TF and NUR-77 are shown to indicate the close sequence homology in the different binding sites. Figure adapted by G. Styger from [58].

B.) The effect of SF-1 on tissue specific expression

The fact that SF-1 protein is widely expressed in steroidogenic tissues led many to believe that it played a role in tissue-specific expression of the various steroidogenic enzyme genes. Some evidence for this exists. Lynch, J.P. *et al.* (1993) [84] found that the SF-1 site in the rat aromatase promoter acts as a cell-selective regulatory element. Other evidence for a tissue-specific role for SF-1 has been found in the human cytochrome P450 side-chain cleavage gene. This promoter was inactive when transfected into nonsteroidogenic cells, but could be activated by the expression of exogenous SF-1 [91]. This indicates that SF-1 protein also plays a role in conferring tissue specificity to this gene. However as it is now known that SF-1 is expressed in many tissues, and not just the steroidogenic tissues, it is clearly not the main factor that determines whether a certain gene is expressed in a certain tissue. The above statement is corroborated by the examples of the gonadotropin genes, LH and FSH. The α-subunit [6, 7] and the LHβ-subunit [63, 64, 65] both have a GSE or gonadotrope specific element, that was thought to confer tissue-specificity to these genes. This GSE element was later identified as a SF-1 DNA-binding site *in vitro*. If SF-1 protein was the only regulator of tissue-specific expression of these genes, they would not only be expressed in the pituitary, but in the adrenals and gonads as well, because SF-1 protein is present in these tissues. SF-1 protein probably acts in combination with other tissue specific factors to confer tissue-specificity. This is certainly the case with the GnRH receptor gene [67].

C.) The relationship between SF-1 and the PKA pathway

We can thus see that SF-1 protein plays an important role in tissue-specific regulation of certain genes. Another area where SF-1 protein seems to be involved is in mediating the PKA response to certain genes, i.e. SF-1 protein

is necessary for the PKA pathway-dependent increase in transcription of certain genes. The mechanism whereby SF-1 protein mediates the PKA response can be either a direct or an indirect effect. The latter would be the case when the PKA pathway changed the levels of SF-1 protein or the affinity of SF-1 for DNA binding. Evidence for this indirect effect was obtained by Shapiro, D.B. *et al.* (1996) [104] when they found that incubation of rat granulosa cells with 8-Br-cAMP (a cAMP analogue) induced a 2-fold increase in SF-1 mRNA. Michael, M. D. *et al.* (1995) [87] found that nuclear extracts isolated from forskolin treated bovine luteal cells, showed a 4-fold increase in binding of a SF-1 DNA-binding sequence probe. This increased binding was unlikely to be due solely to an increase in SF-1 transcription as SF-1 mRNA levels increased by only approximately 2.5-fold over basal. It was postulated that SF-1 protein may be post-translationally modified in response to stimulation of the PKA pathway. Other factors can also increase SF-1 mRNA levels e.g. GnRH. It has been found that GnRH regulates expression of the SF-1 gene in the pituitary, with pulsatile administration of GnRH giving 60% increases in SF-1 mRNA levels [105]. On the other hand, a direct effect would be something like direct phosphorylation of SF-1 by the PKA pathway, leading to increased binding and promoter activity. Transfection experiments done by Zhang, P. & Mellon, S.H. (1996) [93], in mouse Leydig MA-10 and adrenocortical Y-1 cell lines, showed that SF-1 protein can bind to an element in the promoter of the cytochrome P450 17α-hydroxylase/c17-20 lyase gene and that SF-1 mediates the stimulatory effect of the PKA pathway on transcriptional activity. What is more, they showed that SF-1 protein can be directly phosphorylated by PKA at serine and threonine residues. Further work on the direct effect of the PKA pathway on SF-1 was done by Jacob, A.L. & Lund, J. (1998) [94] when they found that mutations in the carboxy terminal AF-2 transactivation core domain of SF-1 dominantly suppressed the PKA-dependent transactivation of the bovine cytochrome P450 17α-hydroxylase/c17-20 lyase gene. It could also be that SF-1 protein is not the only protein involved and that there are cofactors involved e.g. cAMP response element binding (CREB) protein, which is directly targeted for

phosphorylation or dephosphorylation. The mechanism of SF-1 mediation of the PKA pathway on different genes will now be review in more detail.

In addition to the SF-1 DNA-binding site in the rat aromatase gene promoter, Fitzpatrick, S.L. & Richards, J.S. (1994) [85] found a cAMP response element about 80 basepairs upstream and showed that both these elements were required for transcriptional activation by PKA. Later evidence [86] positively identified the protein that bound to this CRE-like element as CREB (CRE binding protein). However the human aromatase gene promoter has been found to be regulated in a different manner compared to the rat promoter. Michael, M.D. *et al.* (1995) [87] found no classical CRE elements in the promoter region and the area crucial for cAMP responsiveness converged exactly with the SF-1 DNA-binding element. They concluded that PKA-stimulated transcription of the human aromatase gene in the ovary is due to increased levels and DNA binding activity of SF-1. Clemens, J.W. *et al.* (1994) [89] found two SF-1 DNA-binding sites in the promoter area of the rat cytochrome P-450 cholesterol side-chain cleavage gene and showed that SF-1 protein indeed bound to these sites. Chau, Y.M. *et al.* (1997) [90] found that simultaneous mutation of these two SF-1 binding sites caused a 7-fold decrease in basal transcriptional activity, but that the decrease in forskolin-stimulated transcriptional activity was only minor. It was therefore postulated that SF-1 does not play a major or direct role in the cAMP or PKA pathway activation of this particular gene.

The other major steroidogenic enzyme gene regulated by SF-1 is the steroidogenic acute regulatory protein or StAR. This protein plays a critical role in the first step in steroid hormone synthesis i.e. the conversion of cholesterol to pregnenolone. Sugawara, T. *et al.* (1997) [95] found a proximal SF-1 DNA-binding element, with the sequence TATCCTTGAC, in the human StAR gene and showed that this sequence confers responsiveness to cAMP in the presence of SF-1. A second more distal SF-1 DNA-binding site was also identified near the transcriptional start site, with the sequence CAGCCTTC, and the same properties as the first site i.e. responsiveness to

PKA. They further found that mutation of either site drastically reduced basal and cAMP-stimulated promoter activity, but that the more proximal site had more pronounced effects. Therefore both SF-1 sites seem to be required for both basal and cAMP-activated StAR promoter activity. A third SF-1 DNA-binding site was found further upstream but was not implicated in the PKA response. Exactly the same results were also obtained when the rat [96] and mouse [97] StAR gene promoters were investigated. Further studies done by Clark, B.J. & Combs, R. (1999) [98] on the human promoter indicated that transcriptional activation of the StAR gene by a SF-1-dependent mechanisms could represent a common pathway for ACTH (via the PKA pathway) and angiotensin II action in stimulating steroid production in both the adrenal fasiculata and glomerulosa. Evidence was also found that SF-1 plays a role in mediating the cAMP regulation of the high density lipoprotein receptor, which mediates the uptake of cholesterol and cholesteryl esters; the substrates for steroidogenesis [99].

In conclusion it would seem that the exact mechanism whereby SF-1 protein mediates the PKA response differs from gene to gene and can even differ for the same gene between species. In the rat aromatase gene SF-1 protein and CREB protein seem to work together to mediate the PKA response, whilst the human aromatase gene contains no CRE elements and the PKA response is mediated by SF-1 protein alone. The rat cytochrome P-450 cholesterol side-chain cleavage gene on the other hand contains two SF-1 DNA binding sites, but neither of them play any role in mediating the PKA response. The PKA response in the rat, mouse and human StAR gene are all mediated by their two SF-1 DNA-binding sites. The one conclusion that can be drawn from all the above evidence is that although it is very difficult to predict the exact mechanism whereby SF-1 mediates the PKA response in a certain gene, for several genes there is strong evidence that it plays a direct or indirect role.

D.) Interaction between SF-1 and other proteins: protein-protein interactions

We can thus see that SF-1 protein controls a wide array of genes, both those involved in steroidogenesis and other functions. In some cases it has been shown that SF-1 protein directly interacts with other proteins to regulate the expression of a certain gene. Montè, D. *et al.* (1998) [91] found that SF-1 protein interacts with exogenous p300 and CREB-binding protein (CBP) in the human cytochrome P450 side-chain cleavage gene. These are closely related ubiquitous coactivators that possibly bridge transcriptional activators and the components of the basal transcriptional apparatus. Another protein that was found to directly interact with SF-1 is the early growth response-1 (ERG-1) protein. Dorn, C. *et al.* (1999) [65] found that the synergy between ERG-1 and SF-1 is required for activation of the LH β-subunit gene by gonadotropin-releasing hormone. They also found that the direct protein-protein interaction requires the zinc-finger domain of ERG-1. One of the best known examples of a protein that interacts directly with SF-1 protein is the orphan nuclear receptor DAX-1 (see section 5 F) [106].

E.) Competition for DNA-binding sites between SF-1 and other proteins

Some proteins compete with SF-1 for the same or overlapping DNA-binding sites. Bakke, M. & Lund, J. (1995) [92] identified an element in the bovine cytochrome P450 steroid 17α-hydroxylase gene that contained the repeated sequences AAGTCA and AGGTCA interspersed by six nucleotides. SF-1 was shown to bind to this sequence and was able to mediate the PKA-stimulated transcription. However another orphan nuclear receptor, chicken ovalbumin upstream promoter transcription factor or COUP-TF also bound to this sequence and repressed SF-1-dependent activation of the gene. This was accomplished by the fact that the two receptors bound in a mutually exclusive manner to the above element. The oxytocin receptor also contains these six basepair repeats and it was found that SF-1 was bound to this element when

the promoter was active, but that COUP-TF occupied this site when the promoter was inactive [103]. The above scenario also takes place in the stimulation of cytochrome P450 aromatase promoter activity in endometriosis tissue and its inhibition in endometrium tissue, due to the competitive binding of SF-1 and COUP-TF to the same *cis*-acting element [88].The competitive binding of SF-1 and COUP-TF seems to be a general way of regulating or controlling the effects of SF-1, where binding of SF-1 causes transcriptional activation and the binding of COUP-TF to the same element causes the promoter to become inactive. The exact mechanism is still unknown, but one can postulate that the relative concentrations of the factors could be regulated to determine which factor binds predominantly.

F.) The role of DAX-1

No discussion on SF-1 control can be complete without a discussion on DAX-1. DAX-1 is also an orphan nuclear receptor and was first discovered and isolated as the gene that caused X-linked adrenal hypoplasia congenita (AHC) and hypogonadoptropic hypogonadism (HHG), both diseases with phenotypes similar to those of SF-1 knockout animals [74, 108]. DAX-1 thus stands for Dosage-sensitive sex reversal-Adrenal hypoplasia congenita critical region on the X chromosome. It is a novel member of the nuclear receptor superfamily, because it lacks the typical zinc finger DNA binding motifs. The demonstration of DAX-1 expression in the hypothalamus and pituitary and the phenotypical similarities between SF-1 knockout and DAX-1 mutation suggested that SF-1 and DAX act sequentially in a hierarchical pathway or that the two proteins interact directly to regulate target genes. The latter hypothesis was proven to be true when Ito, M. *et al.* (1997) [106] showed that coexpression of SF-1 and DAX-1 led to an inhibition of SF-1-mediated transcription. They also showed with *in vitro* protein binding studies that DAX-1 interacted directly with SF-1, but that the ability of SF-1 to bind to DNA was not affected. Further investigation by Crawford, P.A. *et al.* (1998) [109] showed that this interaction occurs through a repressive domain within

the carboxy terminus of SF-1. Also they demonstrate that DAX-1 recruits nuclear receptor co-repressor N-Cor to SF-1. However Lalli, E. *et al.* (1998) [110] could not detect an *in vivo* association between DAX-1 and SF-1 and instead found that DAX-1 binds to hairpin DNA structures. Deletion of these structures removed DAX-1 capacity to repress transactivation by SF-1. They further propose a model whereby the binding of DAX-1 to DNA results in the down-regulation of SF-1-mediated transactivation by recruitment of the transcriptional silencing domain residing in the DAX-1 C-terminus. It is difficult to reconcile these two divergent findings. It is however possible that the multiple SF-1 DNA-binding sites (most genes regulated by SF-1 have at least two of these SF-1 DNA-binding sites situated around 180-200 base pairs from each other) could associate with each other to form such DNA hairpin structures. This may enable DAX-1 (if present) to bind to the DNA secondary structure and simultaneously bind to the SF-1 proteins bound to the DNA, thus repressing the transactivation by SF-1.

6. The role of 25-hydroxycholesterol

As seen above, SF-1 plays diverse roles in regulating various steroidogenic and other genes through out the HPG/HPA axis. The unresolved question however still remains: Is SF-1-dependent transcriptional activity modulated by a ligand, either endogenous to steroidogenic cells, or supplied in an endocrine fashion? Lala, D.S. *et al.* (1997) [111] showed that 25-, 26- or 27-hydroxycholesterol, all suppressers of cholesterol biosynthesis, increased SF-1-dependent transcriptional activity. The activation is dependent upon SF-1's AF-2 domain and it is specific for SF-1. Recently LXR, another nuclear receptor was shown to be responsive to 22-hydroxycholesterol and these results suggest that oxysterols may mediate transcriptional activation via different intracellular receptors [112]. However these previous experiments were performed in non-steroidogenic cells. Mellon, S.H. & Bair, S.R. (1998) [113] used a mouse steroidogenic Leydig cell line MA-10 to determine if 25-

hydroxycholesterol could act as ligand in this milieu. The results indicated that oxysterols are not ligands for SF-1 in these cells and that transcription of six different SF-1-responsive genes was unaffected by the presence of 25-hydroxycholesterol. The question however still remains as to the role that these oxysterols play in non-steroidogenic tissues, i.e. the pituitary and the hypothalamus. It is still possible that SF-1 is regulated differently in different tissue types, and that there might be more than one, yet to be discovered, ligand that controls SF-1-dependent transcription in steroidogenic cells.

CHAPTER 2

MATERIALS AND METHODS

1. Buffers and reagents

All reagents and buffers were made up with distilled water or deionised (MilliQ) water and sterilised by autoclaving or sterile filtration. All materials i.e. powders and organic solvents used were analytical grade, unless stated otherwise.

2. Plasmids

A genomic clone of the mouse GnRH receptor gene was obtained from Stuart Sealfon at Mt. Sinai in New York. This clone contained 1.1 kb of the GnRH receptor gene cloned into the pBluescript®SK phagemid. After mutagenesis of the ATG start codon to a *Bgl II* site, a 580 bp *Bgl II/BamHI* fragment, spanning from -580 to +1 rèlative to the ATG codon, was excised and cloned into the *Bgl II* site of the promoterless luciferase (LUC) reporter plasmid, pGL2-basic (Promega) [58] to yield plasmid LG. The putative SF-1-like site 1 was also mutated from TGTCCTTGG to TGTTTTTGG by Carmen Pheiffer [58] to yield plasmid LGM1. The β-galactosidase expression vector pCISLACZ, used as a control for transfectional efficiency, was provided by Dr. Sue Kidson, Department of Anatomy and Cell Biology, University of Cape Town Medical School. This plasmid has the lacZ gene (3.8 kb) cloned into the *XhoI/SmaI* site of the 6.27 kb pCIS vector. The GnRH-R plasmid (R10) which contains the full length cDNA for the mouse GnRH receptor (1.2 kb), cloned into the *EcoRI/XhoI* site of the cDNA I/Amp vector (4.8 kb), was obtained from Janet Hapgood, University of Stellenbosch (originally from R.P. Miller, Department of Chemical Pathology, University of Cape Town Medical School). Human β-actin cDNA (2.1 kb) cloned into the *BamHI* site of the 2.9

kb ampicillin resistant expression vector Okayama-Berg [114] was provided by Iqbal Parker, Department of Medical Biochemistry, University of Cape Town Medical School.

3. Oligonucleotides

The sequences of the oligonucleotides used are listed below:

Oligonucleotides used for mutagenesis and sequencing

GL1	5' TGTATCTTATGGTACTGTAACTG 3'
GL2	5' CTTTATGTTTTTGGCGTCTTCCA 3'
115	5' AGGGGCTACGGTTACACTGCAGCTTCAGGAGGGCTTGGC 3'
116	5' GCCAAGCCCTCCTGAAGCTGCAGTGTAACCGTAGCCCCT 3'

4. Plasmid isolation

4.1 Extraction of plasmid DNA by the Wizard Maxiprep DNA Purification system

The protocol used was in accordance with the manufacturer (Promega). A single colony of transformed bacteria (see section 12) was used to inoculate 10-30 ml of Luria broth (LB, see appendix A). These starter cultures were incubated overnight at 37 °C in the presence of 50 μg/ml ampicillin. The following day 500 ml of LB was inoculated with 10 ml of the starter culture and incubated at 37 °C overnight with constant agitation in the presence of 50 μg/ml ampicillin.

100 - 500 ml cells were pelleted by centrifugation at 5 000 x g for 10 minutes at 22° - 25° C. The cell pellet was completely resuspended in 15 ml Cell Resuspension Solution (50 mM Tris-HCl (pH 7.5), 10 mM EDTA, 100 μg/ml

RNase A), with the aid of manual disruption. The cells were lysed by adding 15 ml of Cell Lysis Solution (0.2 M NaOH, 1% (w/v) SDS) and inverting the tube for up to 20 minutes until it became clear and viscous. Finally 15 ml of Neutralisation Solution (1.32 M Potassium Acetate (pH 4.8)) was added, the centrifuge tubes inverted several times to mix the solutions, and centrifuged again at 14 000 x g for 15 minutes at 22-25 °C.

The cleared supernatant was filtered through Whatman filter paper into a measuring cylinder to determine the supernatant volume. Room temperature isopropanol (0.5 vol.) was added to the supernatant, the tubes once again inverted several times to mix the contents. The solution was again centrifuged at 14000 x g for 15 minutes at 22-25 °C. The supernatant was discarded and the pellet resuspended in 2 ml TE buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA). 10 ml of DNA Purification Resin was added to the DNA solution and the mixture transferred to a Wizard Maxicolumn coupled to a vacuum source. A vacuum was applied and the solution was pulled through the column. After this step the column was washed with 25 ml Column Wash Solution (80 mM Potassium Acetate, 8.3 mM Tris-HCl (pH 7.5), 40 μM EDTA) and rinsed with 5 ml of 80% (v/v) ethanol. The vacuum was applied for an additional minute to allow the column to dry.

The maxicolumn was inserted into a 50 ml screw-cap centrifuge tube and centrifuged in a swinging bucket rotor at 1300 x g (2500 rpm) for 5 minutes at room temperature. The column was dried for 5 minutes by re-applying the vacuum and then placed in a provided 50 ml screw-cap centrifuge tube. 1.5 ml of preheated (65° C) water (or TE buffer [115]) was added to the maxicolumn. After 1 minute the plasmid DNA was eluted into the tube by centrifuging at 1 000 x g for 5 minutes. The concentration of the DNA was calculated by measuring the OD (optical density) on a spectrophotometer at 260 nm, using the fact that 50 μg of pure DNA would give a reading of 1 at 260 nm. The purity of the DNA was also assessed by determining the ratio of absorbance at 260 nm over the absorbance at 280 nm. The ideal should be

1.8 and if it is lower it means that there is protein contamination. Plasmid preparations were subjected to restriction enzyme digestion (see section 5) to linearise the plasmid. This linear and an uncut sample was run on an analytical agarose gel (see section 6) to confirm the sizes of the plasmids and whether the plasmid was in the supercoiled conformation.

4.2 Extraction of plasmid DNA by the Wizard Midiprep DNA Purification System

The protocol used was in accordance with the manufacturer (Promega). The isolation of plasmid DNA from bacterial cells using the Wizard Midiprep DNA Purification System was in essence performed in the same way as the Maxiprep method described in section 4.1. The volumes of the starter cultures ranged from 10-100 ml and the reagent volumes were also in the range of 3 ml instead of the 15 ml used in section 4.1. The volumes were thus smaller and centrifugation steps could be performed on a bench top centrifuge.

5. Restriction enzyme digestion of DNA

Restriction enzyme digestion of DNA was performed with various restriction endonucleases in the presence of their appropriate buffer, supplied by the manufacturer (Boehringer Mannheim, Roche). As the restriction enzymes are supplied in a glycerol solution, the volume of enzyme added never exceeded one-tenth of the final volume. Digest samples were incubated for 1-2 hours or overnight at 37 °C, unless a different optimum temperature was indicated. Double digests i.e. digestions with two enzymes at the same time, were performed simultaneously if their optimum temperatures and buffer conditions were compatible; otherwise the DNA was first digested with one enzyme, ethanol precipitated, and then digested with the other enzyme. Volumes

varied according to the amounts of DNA that were digested, but normally 5 units of enzyme was added to every 1 μg of supercoiled DNA. Digestion reactions were terminated by adding an equal volume of 2x concentrated gel loading buffer (see section 6) and analysed on an analytical agarose electrophoresis gel (see below).

6. Analytical Agarose gel electrophoresis of DNA

0.75 or 1% (w/v) Agarose gels, depending on the size of the fragments involved, were used to determine the sizes of bands after restriction enzyme digestion and as preparative gels to isolate DNA fragments for cloning (see section 12). Preparative and analytical agarose gel electrophoresis of DNA fragments was performed according to the protocols of Sambrook, J. *et al.* (1989) [115].

Agarose powder (Sigma) was weighed in a 250 ml Erlenmeyer flask. The agarose was dissolved in 1 X TAE (40 mM Tris, 30 mM Acetate, 2 mM EDTA (pH 8)) or 1 X TBE buffer (89 mM Tris; 89 mM Borate, 2 mM EDTA (pH 8)). The opening of the flask was covered with plastic wrapping and a few holes punched in the plastic with a needle or other sharp instrument. It was placed into a microwave oven and heated. The flask was swirled around to check that all the agarose was in solution after which ethidium bromide (10 mg/ml) was added to a final concentration of 0.5 μg/ml to enable the visualisation of the DNA fragments under ultraviolet light. Special care was taken when handling ethidium bromide as it is a suspected carcinogen.

The gel solution was allowed to cool down to about 50 °C and was then poured very carefully, to avoid forming bubbles, into a horizontal gel moulding chamber with a suitable comb in it. The gel was allowed to set for about 30 minutes, the comb was removed and the gel chamber was transferred to a horizontal electrophoresis chamber and covered with 1 x TBE or 1 x TAE

running buffer. DNA samples in 1 x loading buffer (0.04 % (w/v) bromophenol blue, 0.04 % (w/v) xylene cyanol, 5 % (v/v) glycerol in water) were loaded into the wells with a pipette, and the gel was allowed to run for about 90 min at 60 - 100 V (less than 10 V/cm of gel); until the bromophenol blue in the loading dye had travelled about ¾ of the length of the gel. The gel was then taken out and, in a darkened room, put on a UV lightbox and photographed. The bright bands on the photo was caused by ethidium bromide (EtBr) intercalating with DNA and absorbing UV radiation. The markers that were used were pBR322 digested with *Hpa II* which gave sizes of 622 bp; 527 bp; 404 bp and 300 bp; as well as λ (23 kb) digested with *Pst I*, which gave a ladder of bands namely 14 kb; 11 kb; 5,1 kb; 4,8 kb; 4,5 kb; 2,6 kb; 2,0 kb; 1,7 kb; 1,2 and 1,1 kb; 800 bp and 500 bp.

7. Separation of DNA fragments by low melting preparative agarose electrophoresis

Low melting agarose preparative electrophoresis was carried out according to the manufacturer's (FMC Bio Products) protocols. Seaplaque agarose was used for separation of DNA fragments of 1000 bp or more and Nusieve agarose for DNA fragments smaller than 1000 kb. Methodologically the preparation and execution of preparative electrophoresis was very similar to analytical gel electrophoresis (see above). Differences included the additional step of putting the setting gel at 4 °C for 30 minutes to promote the setting of the gels. The gels were also electrophoresed at lower voltage of about 5 V/cm of gel in 1 x TAE running buffer. After electrophoresis the gel was taken out and examined with a long wavelength ultraviolet light and the bands of interest cut out with sterile scalpel blades.

8. Purification of DNA fragments from low melting agarose gels

8.1 Wizard PCR Preps DNA Purification System

The protocol of the manufacturer (Promega) was followed. The agarose gel slices containing the DNA fragments of interest were placed in sterile 1.5 ml microcentrifuge tubes and incubated at 70 °C until the gel had melted. 1 ml Resin was added to the melted agarose and carefully mixed, but not vortexed. A Wizard Minicolumn was attached to a syringe barrel and coupled to a vacuum source. The DNA/Resin mixture was pipetted into the syringe barrel and a vacuum applied to draw it into the column. The vacuum was broken and 2 ml 80% (v/v) isopropanol was added to the syringe and the vacuum reapplied. After the solutions were drawn through the column the vacuum was applied for an extra 30 seconds to dry the column. The syringe barrel was removed and the column transferred to a 1.5 ml microcentrifuge tube and spun for 2 minutes at 10 000 x g. The column was transferred to a new microfuge tube and 50 µl of H_2O added. In the case of a small fragment or insert the water was added at room temperature, but for big fragments or plasmids the water was heated to 65 °C. After a one minute wait period the DNA fragments were eluted by spinning the column for 20 seconds at 10 000 x g at room temperature. The DNA solution was stored at -20 °C until later use.

8.2 NucleoSpin Extract 2 in 1 Kit

All steps were performed in accordance with the protocol supplied by the manufacturer (Macherey-Nagel). The components of the various buffers used were not disclosed in the packet information booklet. The DNA fragment was excised from the gel with a sterile scalpel blade and the weight determined before it was transferred to a microfuge tube. For each 100 mg of gel, 300 µl of buffer NT1 was added and the tube incubated at 50 °C for ten minutes with

occasional vortexing. The sample was then loaded onto a NucleoSpin tube and placed in a 2 ml centrifuge tube. The samples were centrifuged for 60 seconds at 6000 x g at room temperature, and the resultant flow-through discarded. The NucleoSpin tube was once again placed into the centrifuge tube and 700 µl of buffer NT3 was added, followed by a 60 second spin at maximum speed. The previous steps were repeated and the flow-through discarded in each case. The NucleoSpin tube was centrifuged once again to remove all of the buffer NT3. The NucleoSpin tube was placed into a clean 1.5 ml microfuge tube and 50 µl of elution buffer NE was added, after which it was once again centrifuged for 60 seconds at maximum speed. The purified DNA fragments were contained in the flow-through and were stored at -20 °C until further use.

9. Mutagenesis

Prior to the start of this study the SF-1-like site 1 of the GnRH receptor gene promoter, situated at -12 relative to the ATG start codon, had already been mutated and inserted into a luciferase reporter vector by Carmen Pheiffer [58]. There thus existed two different constructs: a wildtype GnRH-R promoter reporter construct named LG and a GnRH-R promoter with mutated SF-1-like site 1 named LGM1. A strategy was devised to mutate the second SF-1-like site (at -247 relative to the ATG) of both these constructs which would result in the generation of four different constructs, as shown in figure 3.3, i.e. the two mentioned above as well as a construct with just SF-1-like site 2 mutated named LGM2 and another construct where both SF-1-like sites 1 and 2 were mutated, named LGM1/2.

A Polymerase Chain Reaction (PCR) strategy was developed to mutate the second SF-1-like site by using specially designed mutation primers that would transform the second SF-1-like site into a restriction site for the enzyme *Pst I*. Thus by digestion with this enzyme the success of the mutagenesis could

easily be determined. Firstly the wildtype construct (LG) was subjected to PCR up with the primers GL1 (situated in the polylinker of the pGL2-basic plasmid) and the antisense mutation primer 116. In a separate reaction the wildtype construct was subjected to PCR with primers GL2 and the sense mutation primer 115. The "Expand High Fidelity PCR System" from Roche was used. Reactions were performed in a final volume of 50 µl in the presence of 0.2 mM dNTPs, 2 ng wildtype (LG) plasmid, 1 x Expand buffer already containing $MgCl_2$ and 0.75 µl Expand Enzyme Mix (making the final concentration of the enzyme mix 1.5 x). Primers, either GL1 and 116 or GL2 and 115 were added at a final concentration of 0.4 µM. The mixture was spun down and the PCR cycle was as follows: 2 minutes at 94 °C, followed by ten cycles of 15 seconds at 94 °C, 30 seconds at 55 °C and 45 seconds at 72 °C. The next twenty cycles consisted of 15 seconds at 94 °C, 30 seconds at 55 °C and 1 minute at 72 °C. The last incubation period was 7 minutes at 72 °C. The PCR was performed in a Stratagene RoboCycler. Two products were formed, the product from GL1 to 116 was 412 bp long and another product of 297 bp was formed from 115 to GL2. These DNA fragments were gel purified in low melting preparative agarose and then purified with the Promega Wizard PCR Preps DNA purification system. The next step was the fusion of the GL1/116 and GL2/115 products. The areas around the 116 and 115 primers were complementary to each other and would thus act as primers for the fusion reaction. Two different amounts of templates were used i.e. 300 ng or 50 ng. Templates were mixed with a final volume of 30 µl in the presence of 0.2 mM dNTPs, 1 x Expand buffer with $MgCl_2$ and 1.5 x Expand Enzyme mix. After mixing and spinning down the reactions were subjected to PCR for thirty cycles as follows: 30 seconds at 94 °C followed by 30 minutes at 72 °C. The product was examined on an agarose gel and found to be too little for further cloning experiments. The PCR product was therefore reamplified with the GL1/GL2 primers by adding 10 µl of the PCR product so that the final reaction volume was 50 µl. The reaction was performed in the presence of 0.2 mM dNTPs, 1 x Expand buffer with $MgCl_2$, 1.5 x Expand Enzyme mix, and 0.4 µM each of the primers GL1 and GL2 using the same cycle program as

above. After checking the product on an analytical agarose gel, the rest of the product was purified on a low melting agarose gel and Wizard PCR Preps DNA purification system. It was then ethanol precipitated by adding $^1/_{10}$ volume (v/v) ammonium acetate and $2^1/_2$ volume (v/v) ethanol and keeping it at -20 °C overnight. The DNA was then centrifuged for 30 minutes at 20 000 x g at 4 °C, followed by a 70 % ethanol wash and finally dissolved in 8 µl H_2O. The next steps involved the restriction digestion of the PCR product as well as the reporter vector and the subsequent gel purification, ligation of the PCR product and the reporter plasmid (section 10) and transformation of the ligated construct into bacterial cells (section 12) and the screening of colonies (section 13) to ascertain whether the colonies did in fact contain the newly made mutated construct i.e. LGM2. The DNA was also sent for sequencing to the DNA sequencing facility at the University of Stellenbosch. The whole procedure was then repeated with the other LGM1 GnRH-R construct to yield LGM1/2 (see figure 3.3.).

10. Ligation

The ligation reaction was done with reagents and solutions from the pMOS kit (Roche). A molar ratio of 5:1, insert to vector was used. 49 ng of insert (mutated GnRH-R promoter region) was ligated into 90 ng of vector (pGL2-basic luciferase reporter plasmid) in a final reaction volume of 7 µl, in the presence of 1 x T4 ligase buffer (Roche), 8 mM DTT, 10 mM ATP, 0.5 µl T4 DNA ligase (Roche) and 4.5 µl H_2O. The mixture was incubated overnight at 16 °C in the Stratagene RoboCycler.

11. Making electrocompetent cells

DH5α (a kind gift from A. Swart, Department of Biochemistry, University of Stellenbosch) and JM109 (Promega) *Escherichia coli* cells were plated out on Luria broth (LB, see appendix A) agar plates and grown overnight at 37 °C. Starter culture flasks with 50 ml SOB (appendix A) were inoculated with above mentioned bacteria and incubated in a shaking incubator overnight at 37 °C. The next day a flask with 500 ml of SOC (Appendix A) medium was inoculated from these starter cultures and incubated for 2-3 hours at 37 °C with constant agitation until the optical density at 550 nm was 0.8 units. The culture flask was put on ice to cool down and all subsequent steps were performed on ice to ensure the highest possible transformation efficiency. The cells in the culture flask were transferred to a centrifuge bottle and pelleted by centrifugation at 2 500 x g at 4 °C for 15 minutes. After discarding the supernatant the pelleted cells were resuspended in 200 ml ice-cold sterile distilled H_2O. After a repetition of the preceding washing steps the cells were washed in ice-cold sterile 10 % (v/v) glycerol. The cells were resuspended in ice-cold sterile 10 % (v/v) glycerol so that the optical density at 550 nm was 100 to 300 units. The electrocompetent cells were aliquoted into prechilled microfuge tubes and snap frozen on dry ice before storage at -80 °C.

12. Transformation of electrocompetent cells

Electrocompetent DH5α or JM109 *E. coli* cells were transformed by electroporation using the Savant GTF 100 Gene Transformer. Electrocompetent cells were carefully removed from storage at -80 °C and thawed on ice. All cuvettes and microfuge tubes were also chilled on ice. 100 ng of DNA in a total volume of 2.6 µl was added to 40 µl electrocompetent cells and gently mixed. The cell/DNA mixture was quickly pipetted into a chilled electroporation cuvette, the lid closed, and transferred to the slot in the electroporator. Care was taken to remove all bubbles in the solution as

this would cause arcing and incomplete transformation. A pulse of 1800 V was passed through the solution. The sample was quickly removed and 1 ml of room temperature SOC medium (see appendix A) added, followed by an incubation period of 1 hour at 37 °C with shaking. After incubation 50 µl of the sample was plated out onto LB-agar plates with the appropriate antibiotics. The rest of the solution was spun down, the supernatant removed, and resuspended in 50 µl SOC medium. This was also plated out onto LB-agar plates and the plates were placed at 37 °C overnight so that transformed colonies could grow. Transfectional efficiency was calculated at 2.6 x 10^6 cfu/µg DNA.

13. Screening of colonies

It was important to screen the colonies formed on the LB-agar plates after transformation to certify that the colonies contained the insert and were not the result of self-ligated vector. This was done by a PCR screening method using the GL1 and GL2 primers situated in the polylinker. From the size of the PCR product it would be possible to determine whether the correct insert was present. Colonies were picked off the LB-agar plates with sterile toothpicks and used to inoculate 20 µl sterile H_2O. 5 µl of this bacterial suspension was added to make a final reaction volume of 20 µl, containing 0.25 mM dNTPs, 1 x PCR buffer (Promega), 1 µM each of GL1 and GL2, 1.5 mM $MgCl_2$ and 0.5 µl Taq Polymerase (Promega). The mixture was mixed well and 50 µl of mineral oil added. The PCR cycle was as follows: 35 cycles of: 1 minute at 94 °C, 1 minute at 50 °C and 1 minute at 72 °C. The final incubation step was 10 minutes at 72 °C. The products were seperated by agarose gel electrophoresis. The rest of the bacterial suspension was used to make glycerol stocks: 400 µl LB medium with ampicillin added at 50 µg/ml, were incubated by the samples and incubated overnight at 37 °C. The next morning 400 µl of 80% (v/v) glycerol was added and the stocks stored at -80 °C.

14. Radioactive labelling of DNA

14.1 Labelling using the Megaprime™ DNA labelling system

The Megaprime™ DNA labelling system (Amersham) uses random sequence hexanucleotide primers to attach to numerous sites along the length of a denatured template DNA. This primer-template complex is a target for the Klenow fragment of DNA polymerase I. New DNA is synthesised in the presence of a radiolabelled nucleotide. [α-^{32}P]-dCTP nucleotides were used in labelling reactions and all necessary safety precautions [116, 117] were followed. Laboratory coats and gloves were worn at all times when handling the radioactive material. A Geiger counter was strategically positioned to monitor any spills during the experimental procedure. All work was done behind a perspex screen and the area and person was regularly monitored for contamination.

DNA fragments to be labelled were treated by restriction enzyme digestion and separation from their vectors by preparative agarose gel electrophoresis (see sections 5 & 7), followed by purification from the gel (section 8). The GnRH-R cDNA was excised by restriction digestion with *EcoRI* and *XhoI* resulting in a fragment of 1.2 kb. The β-actin plasmid was digested with *BamHI* and the cDNA insert was 1.9 kb in size. The Megaprime™ labelling protocol of the manufacturer (Amersham) was followed. 25 ng of DNA and 5 µl of Primer Solution was made up to a volume of 33 µl with H_2O and denatured for 5 minutes at 95-100 °C boiling waterbath. The sample was then spun down and 1 x Labelling Buffer (dATP, dGTP, dTTP in Tris-HCl (pH 7.5), 2-mercaptoethanol and $MgCl_2$, concentrations not given), 5 µl [α-^{32}P]-dCTP (3000 Ci/mmol, Amersham) and 2µl DNA Polymerase Klenow enzyme (1 unit/µl) were added. The sample was mixed and once again spun down followed by an incubation of an hour at 37 °C. The reaction was stopped by the addition of 5 µl 0.2 M EDTA and the volume made up to 100 µl with TE buffer [115]. After purification to remove all unincorporated nucleotides (see

below) the labelling efficiency and specific activity of the probes were determined by Cherenkov counts obtained in a Beckman Scintillation Counter. The specific activity was usually in the region of 10^8 to 10^9 dpm/μg (disintegrations per minute per microgram DNA labelled).

14.2 Removal of unincorporated radioactive nucleotides

It is important to remove all the unincorporated radioactive nucleotides from the probes to accurately determine the specific activity of the probes. The methodology was adapted from Sambrook, J. *et al.* (1987) [115]. The unincorporated radioactive nucleotides were removed by using Sephadex spin columns. Autoclaved glasswool was used to plug the tip of a sterile 1 ml syringe. Sephadex (G25 or G50, Sigma) was added and allowed to settle until the syringe was filled. 100 μl of TE buffer was pipetted on top of the column and the column was spun for 1 minute approximately 400 x g on a bench top centrifuge. This wash process was repeated 4 times. The syringe was placed in a new centrifuge tube with a microfuge tube at the bottom to recover the sample. The radiolabelled DNA solution was then added on top of the column and centrifuged for the same time and speed. The purified radiolabelled DNA probe had collected in the microfuge tube and was transferred to a fresh tube, whilst the unincorporated nucleotides were trapped in the Sephadex spin column.

15. RNA isolation

RNA was isolated by the Tri-Reagent method described by Chomczynski, P. (1993) [118]. TRIzol™ Reagent (GIBCO BRL/Life Technologies) or TRI Reagent™ (Molecular Research Centre) were used and the protocol from the manufacturers was followed. All work was done in RNase free conditions in a laminar flow hood and with pipettes and equipment used solely for RNA work.

All glassware was baked overnight at 180 °C and most solutions made up with diethyl pyrocarbonate (DEPC 1μl/ml) treated water [115]. Plastic apparatus was soaked in a 3% (v/v) H_2O_2 solution and rinsed with RNase-free water. Pipettes were also equipped with filter tips to prevent any RNase contamination. Gloves were also worn at all times and changed regularly.

RNA was isolated from tissue culture cells grown in a monolayer in 10 cm^2 dishes. These monolayer cells were directly lysed in the culture dish, without any washing steps. TRIzol or TRI Reagent was added in the following manner: 1 ml per 10 cm^2 was pipetted onto the monolayer of cells and the dish rotated to ensure complete coverage of the cells. The cell lysate was sucked up and down through a pipette several times until most of the clumps had disappeared and were then transferred to microfuge tubes. The samples were incubated at room temperature for 5 minutes after which 200 μl chloroform was added in a fumehood and the tube vigorously mixed for 15 seconds. Another room temperature incubation step of 2 to 15 minutes followed (the maximum time indicated was always followed) and then the samples were centrifuged for 15 minutes at 12 000 x g at 4 °C. The aqueous phase containing the RNA was transferred to a new microcentrifuge tube and 500 μl of 100% (v/v) isopropanol was added, and mixed thoroughly. Once again the tubes were incubated for 5 - 10 minutes at room temperature followed by centrifugation at 12 000 x g for 10 minutes at 4 °C. The supernatant was discarded and the gel-like pellet containing the RNA was washed with 1 ml 70% (v/v) ethanol and centrifuged for 5 minutes at 7 500 x g at 4 - 25° C. The pellet was air dried at room temperature and dissolved in 50 μl FORMAzol™ (Molecular Research Centre). The concentrations of the RNA samples were calculated by using OD (optical density) readings at 260 nm and using the fact that 40 μg of pure RNA gives an OD reading of 1 at 260 nm. By comparing the ratio of OD at 260 nm relative to the OD at 280 nm the relative purity could be estimated.

16. RNA gel electrophoresis

Analytical and preparative RNA gel electrophoresis was performed in 1% (w/v) agarose gels containing 0.66 M formaldehyde. This protocol was adapted from Sambrook, J. *et al.* (1987) [115] and Ausubel, F.M. *et al.* (1987) [116]. The gel chamber and combs were soaked in 3% (v/v) H_2O_2 solution for an hour before the gel was poured followed by a wash with DEPC-treated water. 1 g Agarose was added to 1 x MOPS buffer (0.1 M MOPS, (pH 7.0), 40 mM sodium acetate, 5 mM EDTA (pH 8)) and 86 ml water. The mixture was boiled in the microwave oven until all the agarose had been dissolved. The gel solution was allowed to cool down to about 50 °C before adding 5.3 ml formaldehyde, making the final concentration of formaldehyde in the gel 0.66 M. The gel was allowed to set for about 60 minutes and then transferred to a gel chamber filled with 1 x MOPS buffer. The buffer volume was just enough to cover the gel by about 2-3 mm.

The RNA samples in FORMAzol were thawed and placed at 55 °C for 10 minutes to ensure complete solubilisation of the RNA. A stock of gel loading cocktail was made containing: 63 µl DEPC-H_2O, 81 µl formaldehyde, 48 µl of bromophenol blue (25 mg/ml) in 50 % (v/v) glycerol and 48 µl 10 x MOPS [44]. For analytical RNA gels 5-10 µg of sample was loaded, but for preparative i.e. gels used for northern blotting, 20 µg of sample was loaded. An equal volume of RNA sample and gel loading cocktail was added and incubated at 55 °C for 15 minutes. In the case of analytical gels 0.5 µl of ethidium bromide (10 mg/ml) was also added to the samples. After the incubation the samples were loaded onto the gel and run for 2 to $2^1/_2$ hours at 60-70 V. In the case of analytical gels the RNA bands were visualised under ultraviolet light.

17. Northern blotting procedure

The procedures followed were modified from those described by Sambrook, J. *et al.* (1987) [115].

17.1 Mono-directional transfer of RNA

This procedure is also known as a capillary blot because it uses capillary forces to transfer RNA from a gel onto a nylon membrane. A rectangular glass dish was filled with 20 x SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0). A glass plate was placed on top of the dish to create a platform or bridge. A wick made from three sheets of Whatman 3MM paper was put over the bridge so that the ends were still hanging into the SSC solution. Care was taken to remove all possible air bubbles by ironing the wick with a sterile rod. The formaldehyde gel (see above) was washed with 2 x SSC and placed on the wick, again making sure that no air bubbles were trapped beneath it. The whole apparatus was covered with cling wrap to prevent the blotting buffer from being absorbed from anywhere but through the gel. The cling wrap covering the gel was cut away with a sterile scalpel.

A sheet of Hybond-N^+ membrane (Amersham) was cut to the exact size of the gel and soaked for 5 minutes in 2 x SSC, before it was placed onto the gel. Air bubbles were squeezed out with a sterile rod. On top of the membrane three pieces of Whatman 3MM filter paper were placed, after they too had been wet in 2 x SSC. A stack of folded absorbent towels about 5 cm high was placed on top of this and lastly another glass plate was put on top followed by a weight of about 0.5 kg. The apparatus was allowed to transfer overnight. The next morning the apparatus was carefully dismantled and the positions of the wells marked on the membrane with a pencil before it was removed. The membrane was washed briefly in 2 X SSC to remove any agarose and placed on filter paper to air dry for 30 minutes. The membrane was then placed RNA side up into a UV crosslinker (Amersham) and exposed for 12 seconds at 312

nm wavelength to fix the RNA onto the membrane. The membrane was then subjected to the hybridisation step immediately or could be wrapped in cling wrap and stored at 4 °C until hybridisation.

17.2 Hybridisation

Hybridisation procedures were carried out with DIG-Easy Hyb solution (Boehringer Mannheim, Roche) and the protocols of the manufacturer were followed. An appropriate amount (20 ml/100 cm^2 of membrane) of hybridisation solution was prewarmed at the optimum hybridisation temperature (50 °C in this case). The membrane was incubated in the pre-Hyb solution for at least 30 minutes with gentle agitation in a hybridisation oven at 50 °C. Meanwhile the ^{32}P radiolabelled DNA probe was denatured by boiling or heating at 95 °C for 10 minutes followed by a rapid cooling on ice. The denatured probe was added to the hybridisation solution (at least 10 ml/100 cm^2) and mixed. As the specific activity of the probes ranged from 10^8 to 10^9 dpm/μg for 100 μl, the final concentration in 50 ml of hybridisation solution was in the region of 10^5 to 10^6 dpm/μg. The pre-Hyb solution was poured off and kept for later reuse and the probe/DIG-Easy Hyb solution was added to the membrane. This was incubated overnight at 50 °C with gentle agitation.

The next morning the Hyb solution was removed and a series of washing steps took place. The membrane was first washed twice for 5 minutes in ample 2 x SSC; 0.1 % (v/v) SDS at room temperature. Then the membrane was washed twice for 15 minutes in ample 0.1 x SSC; 0.1 % (v/v) SDS at 50 °C with gentle agitation. After each washing step the membrane was checked with a Geiger counter to establish how "hot" the membrane still was. After the last wash the excess fluid was blotted off and the membrane wrapped in cling wrap, ready for detection by autoradiography. When necessary the autoradiograph was stripped, i.e. the radioactive probe was removed, as

follows: The membrane was removed from the clingwrap and placed in a plastic dish. A solution of boiling 1% (w/v) SDS was poured over the membrane and it was agitated on a rotary shaker for 2 - 4 hours to strip off all the radioactive probe. It was then taken from the stripping solution and put into a pre-hyb solution to commence reprobing.

18. Autoradiography

Membranes were probed with [α-^{32}P]-dCTP labelled DNA probes (see above) and exposed to HYPERfilm™ (Amersham) in a lightproof exposure cassette at -70 °C, with an intensifying screen, for time periods ranging from 1 hour to 1 week, depending on the specific activity and age of the probe. The cassette was taken out of the -70 °C freezer and allowed to thaw to room temperature. In a dark room with only red-light illumination, the film was taken out and placed in developing solution (Cronex, Protea Medical Services) for approximately 2 minutes. The film was removed with plastic tweezers and rinsed under running tap water until all the developer had been washed off. The film was then placed into a fixing solution (Cronex, Protea Medical Services) for another 2 minutes, followed by another rinse, after which the film was hung up to air dry. To quantify the radiolabelled probe signals on these films, the films were scanned using a densitometer (Shimadzu, Dr. Gene Roussou, University of Cape Town) and relative values were given based on the intensity of the various bands. These values were then used to construct diagrammatic histograms (see results), using the Graphpad Prism computer program.

19. Tissue culture

Immortalised αT3 cells from mouse pituitary gonadotropes [24], a kind gift from Pamela Mellon, Regulatory Biology Laboratory, The Salk Institute, La

Jolla, California, were used in all experiments. The αT3 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, Gibco BRL) supplemented with 1 g/L glucose. The DMEM was also supplemented with 10% (v/v) fetal calf serum (FCS, Highveld Biological) and 1U/ml Penicillin-Streptomycin (Gibco BRL, 100U/ml). The fetal calf serum was heat inactivated before use by heating it to 50 °C before freezing it at -20 °C. Cells were grown in sterile polystyrene 75 cm^2 culture flasks (Pyrex, Falcon or Corning) or 100 mm dishes (Corning) with 10-12 ml of medium and incubated at 37 °C with 5% CO_2. The medium was removed every 4-5 days and replaced with fresh media in a sterile hood using sterile pipettes.

When cells became confluent, they were passaged by removing the medium and adding 3-5 ml of a trypsin solution (see appendix A) to the flask. The flask was then replaced into the incubator and left for an additional 5 - 10 minutes. After this incubation time, most of the cells were no longer attached to the flaskbed and floating in the liquid. Some cells were still attached to the bottom and were removed by gently tapping on the side of the flask with one hand. By using an automatic pipette the liquid was sucked up and down to wash off any cells still clinging to the flask; as well as breaking up any cell clumps. For routine culture splitting, the trypsin solution containing the cells was then transferred into a 50 ml screw-cap centrifuge tube and mixed with about 40 ml of medium. This solution was then added to 4 or 5 new flasks and split 1:4 or 1:5, depending on the confluence and growth rate of the cells, and returned to the incubator. If new flasks were not needed the rest of the cells were discarded or used to make stocks.

For cell counting, and subsequent plating out into multiwell plates or Petri dishes, ± 35 ml of fresh medium was added to the trypsin solution in the centrifuge tube so that the final volume was 40 ml. The cells were spun down at 200-500 rpm in a bench top centrifuge with a swinging bucket rotor for 5 minutes. The supernatant was aspirated and the cell pellet resuspended in a small amount of medium to ensure that all cell clumps were dispersed. More

medium was later added (± 50 ml, depending on the confluency of the flask being counted) and from this suspension 100 µl was removed and added to the same amount of trypan blue (10 mg/ml) in a microfuge tube. The tube was stored on ice for 5 minutes, after which a haemocytometer was loaded by capillary action. The amount of cells in ten squares of the haemocytometer (X) was counted, but cells that were stained blue were not counted as they indicated the dead cells. The concentration of cells were calculated as follows: $[Cells/ml] = \frac{X}{10}$ x 10^4 x 2.

For RNA isolation studies cells were plated out at densities of 2-2.5 x 10^6 cells per 100 mm dish and 10 ml medium, with 10% fetal calf serum, was added. The cells were grown overnight and then test compounds were added in dimethyl sulfoxide (DMSO), at a final concentration of equal or less that 0.1%, and left on for a specified period after which the RNA was isolated. For plating into 6-well plates for transfection experiments see section 20.2.

20. Transfections

20.1 Transfection of αT3 cells with the calcium phosphate method

The calcium phosphate transfection method was adapted from Ausubel, F.M. *et al.* (1987) [116] and Ferguson, C. (personal communication). Cells were plated out at 5 x 10^4 cells per well into 6-well tissue culture plates (Falcon or Nunc). Fresh medium, with 10% fetal calf serum (FCS), was added, 2.5 ml per well, and the cells were allowed to settle overnight. The following day the transfection mix was made containing 12 µg of GnRH-R reporter construct and 2.75 µg β-galactosidase expression vector as control for transfectional efficiency, per well. Since the transfections were performed in duplicate the mix for two wells was combined and thus contained 24 µg GnRH-R reporter construct and 5.5 µg β-galactosidase expression vector. This was added to

20.8 µl 2 M $CaCl_2$ and the volume was made up to 170 µl with water, and this tube was labelled A. In a separate tube, designated B, 1.7 µl phosphate buffer (PO_4 Buffer) (70 mM Na_2HPO_4, 70 mM NaH_2PO_4, pH 7.0) was added to 170 µl 2 x HeBS (HEPES buffered saline, see appendix A). The contents of tube A was added dropwise to tube B, followed by vortexing after each drop and left to incubate at room temperature for 30 minutes until a fine precipitate formed. The precipitate was resuspended and added to the cells in a dropwise manner. The precipitate was left on the cells for 24 hours and then they were twice washed with phosphate buffered saline (PBS) (10 mM Na_2HPO_4, 1.75 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.0)

20.2 Incubation with test compounds

As seen above, after the transfection procedure the cells were washed twice with phosphate buffered saline (PBS) and then fresh medium containing 10% fetal calf serum (FCS), was added. The test compounds were then added and the cells incubated with the test compounds for a specified amount of time. Forskolin (Sigma) and 8-Bromo-cyclic AMP (Sigma) were added in dimethyl sulfoxide (DMSO) and 25-hydroxycholesterol (Sigma) was added in ethanol to the cells. The final concentration of ethanol or DMSO was equal or less than 0.1 %.

20.3 Preparation of cell extracts

After the incubation period with the test compounds was over the cells were ready for preparation of cell extracts. This was done according to the procedures in Promega's 'Luciferase Assay Kit'. The Reporter Lysis Buffer (RLB) supplied with the kit was diluted 4 x with H_2O to make a 1 x RLB solution. Meanwhile the medium was removed from the cells and they were washed twice with PBS before 250 µl of 1 x RLB was added to each well. The

plates were rotated to ensure complete coverage of the cells and left at room temperature for 15 minutes. Cells were then scraped with a rubber policeman or cellscraper and the lysate transferred to a microfuge tube and placed on ice. The tubes were then vortexed and centrifuged for 30 seconds at 12000 x g at room temperature. The supernatant was then transferred to a new tube and allowed to freeze at -80 °C before the assays were done.

20.4 Luciferase assays

Manual luciferase assays were adapted, according to C. Ferguson, from the Promega Luciferase kit manual [58]. 350 µl Tris-acetate buffer (0.1 M Tris-acetate, 2 mM EDTA, pH 7.75 with acetic acid) and 100 µl luciferase assay substrate were added to a cuvette and vortexed before being inserted into a luminometer (1253-003 Luminometer, BioOrbit) and the background reading taken. The adaptation from the kit manual, in regard to the addition of Tris-acetate buffer, was done to ensure an adequate volume in the cuvette. The cuvette was quickly removed and 20 µl of cell extract added, once again vortexed and the luminescence read after 30 seconds. The luciferase values were divided by the β-Galactosidase values (see below) to correct for transfectional error and expressed relative to a control value.

Promega luciferase assays were also done with the automatic Luminoskan RS (Labsystems). In this case only 10 µl of cell extract was pipetted into black 96-well plates (Nunc). The plate was inserted into the Luminoskan, which automatically dispensed 50 µl of luciferase substrate into each well, immediately followed by the reading of the luminescence value. Due to the small area of the wells in the 96-well plate, the addition of Tris-acetate buffer was not necessary.

20.5 β-Galactosidase assays

20.5.1 Spectrophotometric β-Galactosidase assay

Manual β-Galactosidase or β-Gal assays were performed by adding 30 µl of Buffer Z (60 mM Na_2HPO_4, 40 mM $NaH_2PO_4.2H_2O$, 10 mM KCl, 1 mM $MgCl_2$, 50 mM β-mercaptoethanol, pH 7.) to 10 µl cell extract in a microfuge tube [58]. To start the reaction 8 µl of 4 mg/ml of the β-Gal substrate ONPG (O-nitrophenyl-β-D-galactopyranoside (Sigma) dissolved in a solution of 60 mM Na_2HPO_4, 40 mM NaH_2PO_4, pH 7.0) was added and the samples were incubated at 30 °C for 30 minutes or until a yellow colour developed. The reaction was halted by the addition of 20 µl 1 M Na_2CO_3 and the samples spectrophotometrically measured at a wavelength of 420 nm.

20.5.2 Galacto-*Star*™ chemiluminescent β-Galactosidase assay.

The protocol of the manufacturer (TROPIX) was followed. The required amount (100 µl per sample) of Reaction Buffer Diluent (100 mM sodium phosphate pH 7.5, 1 mM magnesium chloride, 5 % Sapphire-II™) was equilibrated to room temperature and used to dilute the Galacto-*Star* substrate 1:50. 10 µl of cell extract was aliquoted into wells of a black 96-well microplate and the plate inserted into a Luminoskan RS (Labsystems) microplate reader. 100 µl of diluted Galacto-*Star* substrate was automatically dispensed into each sample and the plate incubated for 60 minutes. The Luminoskan then read each sample for 1 second to determine the amount of luminescence in each sample.

20.6 Statistical analysis

Luciferase and β-Gal assay results were graphically analysed with GraphPad PRISM™ version 2.1 software from GraphPad Software Incorporation. The

data was analysed with One-way ANOVA as well as Dunnet's multiple comparisons test to determine the statistically significance between experimental differences.

CHAPTER 3

RESULTS AND DISCUSSION

1. Preparation of GnRH receptor promoter constructs

Prior to the start of this thesis, GnRH receptor promoter reporter constructs had previously been prepared by others i.e. the wildtype (LG) and the SF-1-like site 1 mutant (LGM1) constructs (see methods). During the course of the present study two additional constructs were prepared by PCR mutagenesis, where the SF-1-like site 2 of both of the above constructs was mutated to a *PstI* restriction site, yielding LGM2 and LGM1/2. The creation of these novel *PstI* sites, previously absent in the original constructs, made it possible to determine the success of the PCR mutagenesis by restriction enzyme digestion (see figure 3.1). This technique was also used to select bacterial clones containing the correct plasmid insert for growing up for further sequencing. Figure 3.1 shows such a digestion as well as an alternative digestion with *HindIII* which, just like *PstI*, would only cut the constructs once, thereby linearising them.

Lanes 8 to 11 in figure 3.1 panel A contain the undigested GnRH receptor constructs. All the constructs are the same size, i.e. 6.8 kilobases, yet all of them have a major band running lower than 4.1 kb. This is known as supercoiled DNA and because of its tightly wound structure it moves through the gel faster than a linear strand of the same length. The presence of the supercoiled DNA also signifies good quality DNA for transfection. The other, less intense band seen at around 9 kb represents nicked circular plasmids i.e. plasmids that have been cut on one strand and are partially unwound. These bands ran even slower than the linear bands in lanes 2 to 5 which ran at approximately the 6 kb mark.

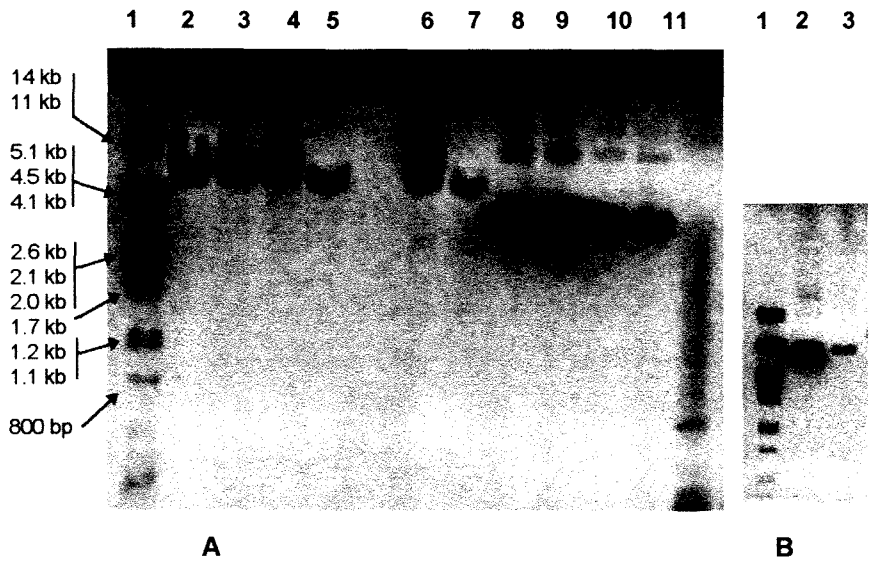


Figure 3.1: Restriction enzyme digestion of the four GnRH receptor promoter constructs. Plasmids were incubated with 5 U of enzyme per μg of plasmid at 37°C for 1 hour or overnight and the digestion products were run on a 0.7% agarose gel at 100V for 80-90 minutes. 3 μg of DNA was loaded for each sample. Panel A: Lane 1 contains a DNA marker i.e. λ DNA digested with *PstI*. Lanes 2, 3, 4 and 5 contain LG, LGM1, LGM2 and LGM1/2, respectively, cut with *HindIII*. Lane 6 contains LGM2 linearised with *PstI* and lane 7 shows the result of the same restriction enzyme on LGM1/2. Lanes 8 to 11 contain the undigested plasmids in the same order as lanes 2 to 5. Panel B: Negative control digestion by *PstI* on LG and PLGM1. Lane 1 is the same DNA size-marker as in panel A lane 1 i.e. λ DNA digested with *PstI*. Although the gels are not on the same scale the marker sizes correspond. Lane 2 contain LG DNA digested with *PstI* and lane 3 the same enzyme digestion of the LGM1 plasmid. The band in lane 3 is less intense than the one in lane 2 due to an error in loading the gel.

HindIII is an enzyme that cuts only once inside the pGLBasic plasmid and not within the GnRH receptor promoter region. Therefore digestion with this enzyme will lead to the linearisation of the plasmids and they will then run according to size, as seen in lanes 2 to 5. In lanes 6 and 7 the newly constructed LGM2 and LGM1/2 plasmids that have been digested with *PstI* can be seen. It is clear that the bands are exactly the same size as those digested with *HindIII*, thereby suggesting that those constructs now contain a SF-1-like site 2 that has been mutated to a *PstI* site. It was also found that *PstI* was unable to cut the LG and LGM1 constructs. In figure 3.1 panel B it can clearly be seen that the LG and LGM1 constructs still ran at the same position as uncut plasmids after incubation with *PstI*, thus showing that the

```
-661  TGTATCTTAT GGTACTGTAA CTGAGCTAAC ATAACCCGGG AGGTACCGAG
-661  TGTATCTTAT GGTACTGTAA CTGAGCTAAC ATAACCCGGG AGGTACCGAG

-611  CTCTTACGCG TGCTAGCTCG AGATCCACTA GTTCTAGAAT AATTGGTATT
-611  CTCTTACGCG TGCTAGCTCG AGATCCACTA GTTCTAGAAT AATTGGTATT

-561  AGAACAGGCT GCTTAAAACA GTTAAAGTAC TAGCTATAAG TCCGTCGTGT
-561  AGAACAGGCT GCTTAAAACA GTTAAAGTAC TAGCTATAAG TCCGTCGTGT

-511  GACTATTCAG CAAAATGCAT TTGAAAAGCA ATTGTTTTGA GAAGTATGGT
-511  GACTATTCAG CAAAATGCAT TTGAAAAGCA ATTGTTTTGA GAAGTATGGT

-461  CTTCAAACAA CAGATTTTAA ATTGGATCGG GATTTTTAAA TTACTTTTCT
-461  CTTCAAACAA CAGATTTTAA ATTGGATCGG GATTTTTAAA TTACTTTTCT

-411  GTATTTCATT TTGTATCTGT CTAGTCACAA CAGTTTTTAG AAAACCTATT
-411  GTATTTCATT TTGTATCTGT CTAGTCACAA CAGTTTTTAG AAAACCTATT

-361  CATTAAGGCT AATTGGATGA TATTATGAGT CACTTTCGAC ATCAGAATTA
-361  CATTAAGGCT AATTGGATGA TATTATGAGT CACTTTCGAC ATCAGAATTA

-311  GACTCCAAGT GTCCTTCCTC ACCTACGATA AAAAGACGGG CCATCTGCTG
-311  GACTCCAAGT GTCCTTCCTC ACCTACGATA AAAAGACGGG CCATCTGCTG

                          2
-261  AGGGGCTACG GTTACACTTG GCCTTCAGGA GGGCTTGGCA TGTTCTGTTA
-261  AGGGGCTACG GTTACACTGC AGCTTCAGGA GGGCTTGGCA TGTTCTGTTA

-211  GCACTCTTTT AGATTATAAA CCGAAAAACA AGTTTACCTT GATCTTTCAC
-211  GCACTCTTTT AGATTATAAA CCGAAAAACA AGTTTACCTT GATCTTTCAC

-161  GTTAAGTCCA GAGTATCTTG GGAAAAATAA ATTAGGCAGA AATGCTAACC
-161  GTTAAGTCCA GAGTATCTTG GGAAAAATAA ATTAGGCAGA AATGCTAACC

-111  TGTGACGTTT CCATCTAAAG GAGGCAGACA TCAACAGCGC TTCGCGTTCA
-111  TGTGACGTTT CCATCTAAAG GAGGCAGACA TCAACAGCGC TTCGCGTTCA

-61   GTTATGATAA AACATCAGAA GTAACAGGGA CTCCACTCTT GAAGCCTGTC
-61   GTTATGATAA AACATCAGAA GTAACAGGGA CTCCACTCTT GAAGCCTGTC

      1
-11   CTTGGAGAAA TAGATCTAAG
-11   CTTGGAGAAA TAGATCTAAG
```

Figure 3.2: DNA sequencing results of the LGM2 GnRH receptor reporter construct, compared to the wildtype sequence. The bottom, red sequence represents LGM2 and the top, blue sequence that of the wildtype LG. The two SF-1-like sites are bold and boxed and the mutations to site 2 can clearly be seen when comparing the sequences. The site 1

sequence however is still identical. The areas in black represent sequences from the reporter plasmid pGL2basic. The area in Italics represents the ATG transcription start site that had previously been mutated to AGA (bold) (see methods). The numbering is relative to the ATG start codon. Figure by G. Styger.

result was specific and not merely a non-specific artefact of the digestion. The restriction digestion evidence was however not enough to prove whether mutagenesis was successful or not, even with the additional evidence that *PstI* was unable to digest LG and LGM1. The only way to positively prove that the LGM2 and LGM1/2 constructs contained a *PstI* recognition site was by DNA sequencing. The results for one of these constructs, LGM2, is compared with the DNA sequence of the wildtype plasmid in figure 3.2. It can clearly be seen that while the SF-1-like sites 1 of these two constructs are identical, there has been a mutation in the site 2 of the LGM2 construct. The same sequencing comparison was also done between LGM1 and LGM1/2 (data not shown) where once again their SF-1-like site 1 was identical (although it was different from LG and LGM2), but their site 2 sequences were different due to the mutation of LGM1/2. The sequencing evidence, together with the restriction digestion results, positively proved that the mutagenesis of the SF-1-like site 2 was successful.

2. The role of SF-1-like sites 1 and 2 of the GnRH receptor promoter in basal transcription

The four constructs above (see figure 3.3) were further used in transfection experiments, where they were transfected into αT3 cells together with the β-galactosidase expression vector, as a control for transfectional efficiency. The cells were then lysed after an incubation period and luciferase and β-galactosidase (β-gal) assays were performed. The values from the luciferase assay were divided by the corresponding β-gal value to normalise the values in terms of transfectional efficiency. For each experimental condition i.e. construct type or incubation condition, duplicate experiments were performed.

The luciferase and β-Gal assays were also done in duplicate for each individual sample. Lastly experiments were repeated several times, to establish significance of the data. To compare the normalised results i.e. luciferase divided by β-Gal, from one experiment with those from another, the average of the two wildtype LG values was calculated and all other values were expressed as a percentage thereof (see table 3.1, 3.2 and 3.3).

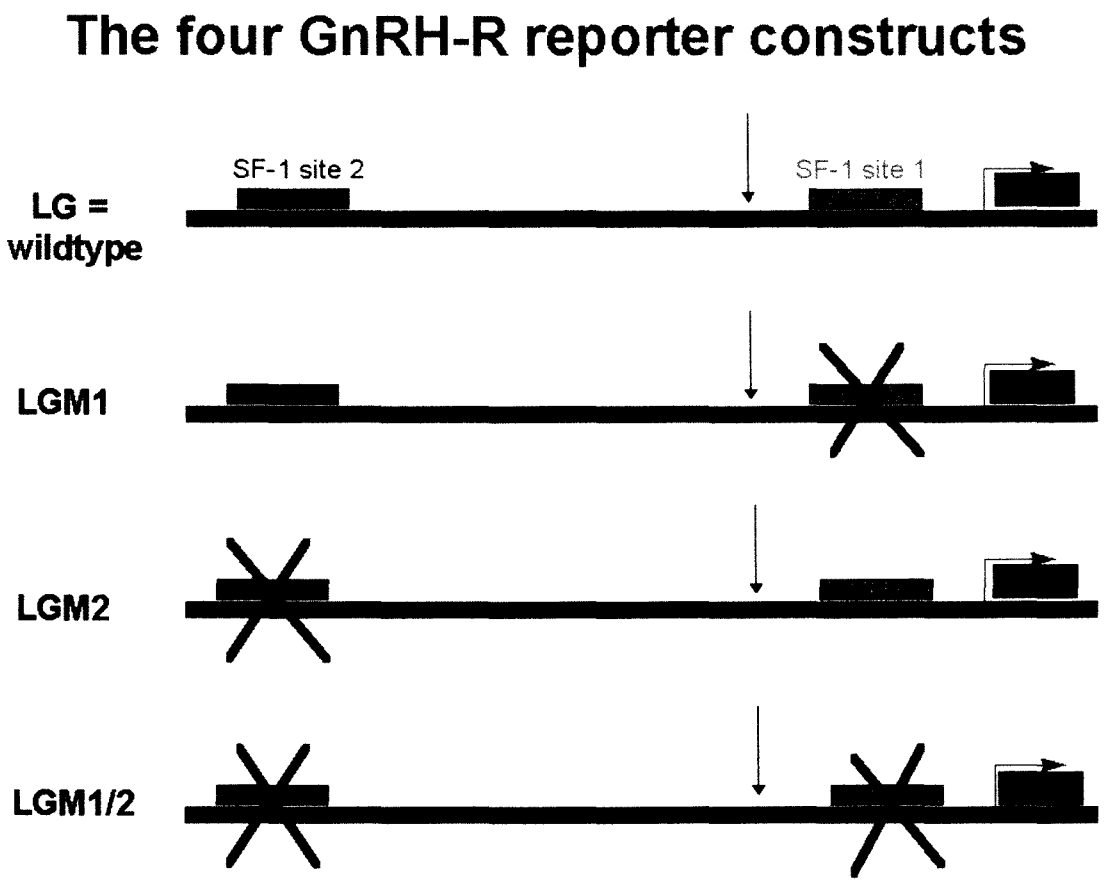


Figure 3.3: Diagrammatic view of the four different GnRH receptor promoter constructs. The promoter region of the GnRH receptor was mutated and four different constructs were created. These were then linked to a luciferase reporter plasmid. See methods (section 9) for experimental detail. The SF-1-like site 1 is located at -14 to -5 relative to the ATG and SF-1-like site 2 is situated at -247 to -238 relative to the ATG start codon. The sequences are as follows: wildtype (wt) SF-1 site 1 = TGTCCTTGG, the mutated (mut) SF-1 site 1 = TGTTTTTCC, the wt SF-1 site 2 = TGGCCTTCA and the mut SF-1 site 2 = GCAGCTTCA. LUC represents the luciferase reporter gene. The black arrow shows the position of the transcriptional start site at -63 relative to ATG. The figure is not to scale and was compiled by G. Styger.

In the first transfection experiment the relative contribution of the two putative SF-1-like sites in the GnRH-R gene promoter to basal expression was investigated. The basal promoter activity of the following four GnRH receptor promoter constructs was tested in αT3 cells: LG (the wildtype construct), LGM1 (site 1 mutated and site 2 unchanged), LGM2 (site 2 mutated and site 1 unchanged) and LGM1/2 (both sites mutated) (see figure 3.3). The results from 3 independent experiments are shown in figure 3.4 (also see table 3.1).

Figure 3.4 shows that mutation of the SF-1-like site 1 (the proximal one) causes a slight increase in transcriptional activity of the GnRH receptor promoter, but this increase was not statistically significant. There was no significant change in promoter activity, which indicates that site 1 does not play a major role in basal expression of the GnRH receptor gene. The mutation of the SF-1-like site 2 (the distal site) also had no significant effect on GnRH receptor promoter transcriptional activity. However there was a significant 50% or 2-fold decrease in reporter gene expression, compared to wildtype, when both SF-1-like sites were mutated. This result was very interesting in light of the evidence that mutation of site 1 alone or site 2 alone had no effect on promoter activity. This evidence with the double mutant indicates that SF-1 protein is indeed necessary for full basal expression, but that only one SF-1-like element needs to be occupied i.e. SF-1 can either bind to site 1 or site 2 to effect basal expression and it is only when it is unable to bind to either site that basal expression is decreased. This evidence further suggests that these sites are somehow positioned close to one another when basal transcription takes place. The sites are approximately 230 bases from each other and it is conceivable that they form an association with each other due to chromatin structure or some other unusual DNA structure.

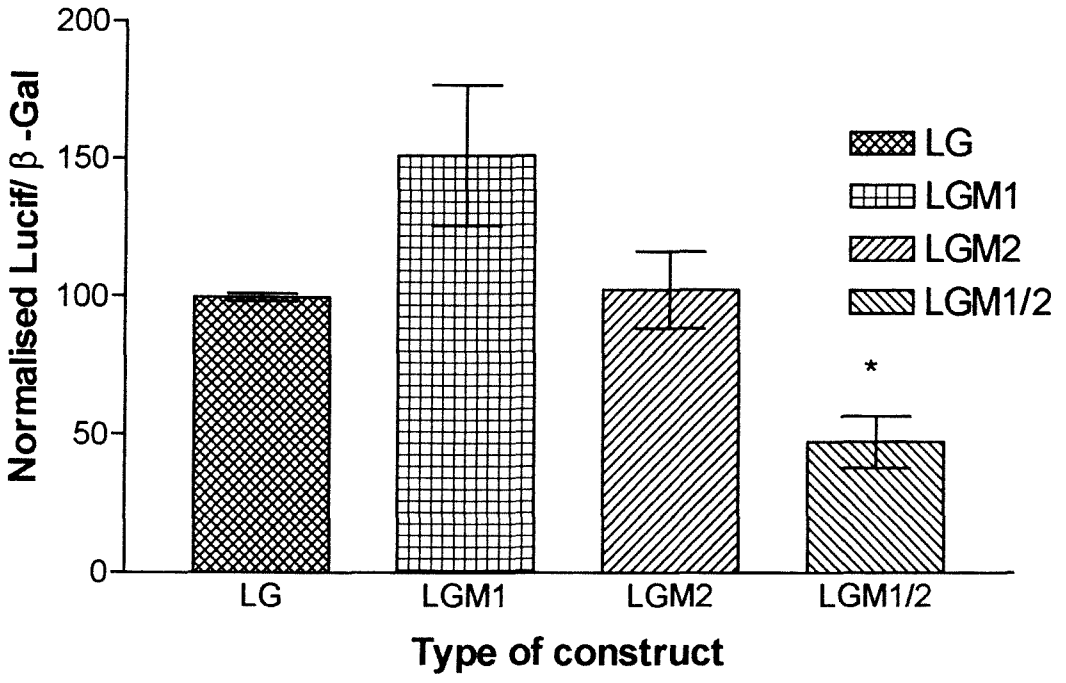


Figure 3.4: Effect of SF-1 site mutations on the basal transcriptional levels of four GnRH-R reporter constructs. αT3 cells were transfected with one of four GnRH-R reporter constructs together with a β-Gal expression vector. Samples were transfected in duplicate and duplicate assays were also done on each sample. The luciferase (Lucif) values were divided by the corresponding β-Gal (β-Gal) value to get normalised expression for each of the samples. The average of the wildtype (LG) was taken as 100% activity and all the other results from this experiment were calculated as a percentage relative to it. Standard errors are shown and the n value is 3. The * represents a P value smaller than 0.05 ($P < 0.05$) relative to LG.

3. Responsiveness of GnRH receptor promoter to protein kinase A (PKA) activation

A.) <u>The effect of PKA on the endogenous GnRH receptor gene</u>

Transient promoter-reporter transfection experiments represent a very artificial or *in vitro* situation, i.e. several copies of the promoter area of a single gene are introduced into cells and this DNA is not integrated into the

Table 3.1: Example of original data from a single representative experiment on cells transfected with either one of four GnRH receptor constructs.

		A	B	C	D	E	F	G	H	I
		Luciferase			β-Galactosidase			Lucif/β-Gal	% TO LG Ave	STDEV
		1	2	Mean	1	2	Mean			
LG	a	0.074	0.084	0.079	0.038	0.051	0.044	1.789	103.9	5.520
	b	0.069	0.119	0.094	0.063	0.050	0.057	1.655	96.11	
LGM1	a	0.142	0.162	0.152	0.062	0.071	0.066	2.294	133.2	49.11
	b	0.105	0.096	0.101	0.098	0.085	0.092	1.098	63.78	
LGM2	a	0.080	0.077	0.079	0.038	0.084	0.061	1.296	75.29	0.742
	b	0.050	0.057	0.054	0.039	0.045	0.042	1.278	74.24	
LGM1/2a		0.035	0.027	0.031	0.092	0.096	0.094	0.330	19.16	19.42
	b	0.065	0.117	0.091	0.057	0.170	0.113	0.803	46.62	

Columns A and B show duplicate luciferase assay results (i.e. 1 and 2) for an individual experiment with a given construct and "a" and "b" are the results from the duplicate cell incubation experiments for each construct. Column C contains the mean of columns A and B. The same applies for columns D, E and F but the values are from β-Galactosidase assays. Column G shows the normalised results, i.e. luciferase divided by β-Gal to normalise for transfectional efficiency. The average of the duplicate control samples, in this case LG, was then taken as 100% and all other results was calculated as a percentage of this value. This is contained in column H. Lastly column I contains the standard deviation between the duplicate values for each construct.

genome. Therefore these transfected reporter constructs do not have the natural chromatin structure that the endogenous gene would have. In an attempt to use a more *in vivo* model, GnRH receptor mRNA levels in αT3 cells were measured after incubation with various compounds. The reason for this was to see if the endogenous GnRH receptor gene is regulated by PKA activation. This was done by incubating αT3 cells with forskolin, an activator of adenylate cyclase which leads to an influx of extracellular Ca^{2+}, or 8-bromo-cyclic-adenosine monophosphate (8-Br-cAMP), both PKA pathway activators, and then isolating total RNA from these cells. The RNA was first run on an analytical gel to make sure that the mRNA was intact and that there was no DNA contamination of the samples (see figure 3.5).

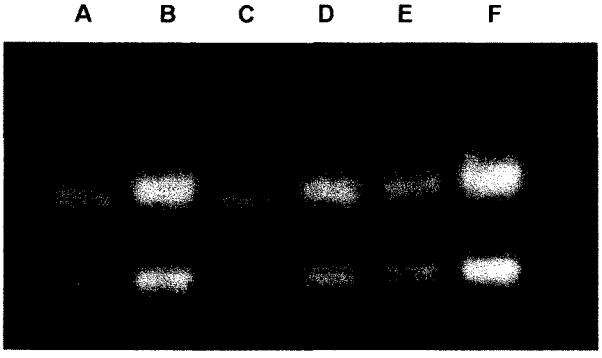


Figure 3.5: Example of an analytical gel loaded with 1 μg of total RNA isolated from αT3 cells incubated with or without various test compounds, showing the two ribosomal bands of 30S and 18S.

Figure 3.5 is an example of an analytical RNA gel and the amount of RNA added for lanes A to F is 1 μg total RNA per lane. However one would still have expected to see bands of the same intensity between samples of the same experiment, clearly this is not the case. This was due to the difficulty in solubilising the RNA in solution. To overcome this problem subsequent total RNA samples were read on the spectrophotometer at 260 nm just after the isolation step. The samples were then stored at -80 °C until they were used for Northern blotting. On the day that the Northern blot was to take place the RNA samples were thawed and incubated for 10 minutes at 55 °C to ensure complete solubilisation of the RNA before they were once again

spectrophotometrically quantified and run on an analytical gel. After this treatment the RNA once again proved to be intact, but the amount of RNA in the different lanes was more equivalent. Unfortunately a picture of this gel was not taken. Thus it is very important to include a β-actin mRNA detection step, because even if the loading amount was different between two samples, the β-actin mRNA normalisation will correct these differences. The RNA samples were then run on a formaldehyde-containing agarose gel and transferred onto a nitro-cellulose membrane before being probed with ^{32}P-labelled cDNA probes for GnRH receptor and β-actin, a constituently expressed housekeeping gene. The same membrane was first probed for GnRH receptor, stripped and then reprobed with β-actin probe. An example of a resulting autoradiograph can be seen in figure 3.6.

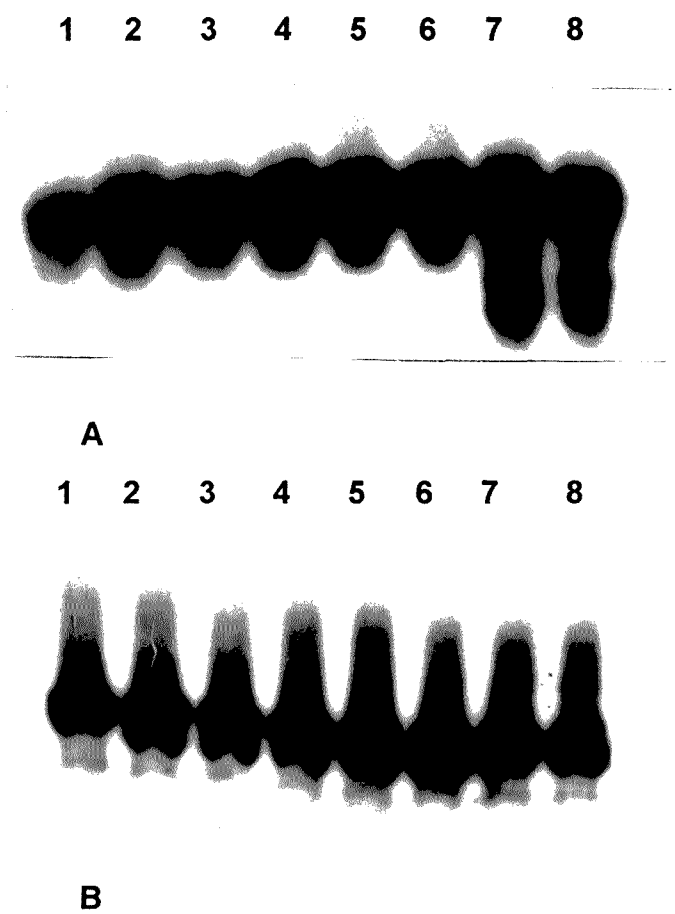


Figure 3.6: Autoradiograph of a single membrane, from one experiment, onto which RNA was transferred and then UV crosslinked. The RNA was isolated from αT3 cells incubated with 1 mM 8-Br-cAMP (lanes 3 and 4), 10 μM forskolin (lanes 5 and 6), 100 μM forskolin (lanes 7 and 8), for 18 hours, as well as cells that were not incubated with any test

compounds and served as controls (lanes 1 and 2). After transfer, the membrane was hybridised with a radioactively labelled ^{32}P cDNA probe for GnRH receptor (panel A). Thereafter the membrane was stripped and reprobed for using the β-actin probe (panel B). The GnRH receptor mRNA bands seen in panel A consist of a major band of 4.5 kb for lanes 1 - 8 and a very minor band of 1.8 kb for lanes 1 - 6. In lanes 7 & 8 the 1.8 kb band is relatively more abundant. The β-actin bands in panel B are 2 kb in size.

It is clear from figure 3.6 that the intensity of the β-actin band (panel B) is similar through-out the experiment. This means that β-actin expression is unaffected by forskolin or cAMP and that it is an ideal control to normalise the results for differences in the total amount of RNA between different samples. Panel A shows the effect that increasing concentrations of forskolin or a single concentration of 8-Br-cAMP have on the endogenous mRNA levels of the GnRH receptor gene. When comparing lanes 3 and 4 i.e. the cells that have been incubated with 1 mM 8-Br-cAMP, with the control lanes 1 and 2, it is clear that there is a small increase in the intensity of the bands in lanes 3 and 4. This means that 8-Br-cAMP does indeed seem to increase GnRH receptor mRNA levels. When one looks at the effects of forskolin on the mRNA levels it also appears to increase the band intensities. Lanes 5 and 6 i.e. 10 μM forskolin, are approximately 1.5 to 2 fold darker (judged by eye) than the control lanes. It is clear that the lanes with RNA isolated from cells incubated with 100 μM forskolin, i.e. lanes 7 and 8 are much more intense than the control bands. It is also interesting to note that the minor band of 1.8 kb is much more intense than in any of the previous lanes. This might mean that under certain circumstances e.g. activation of the promoter by the PKA pathway, alternate transcription start sites are used, resulting in mRNA of a smaller size. What the function of these alternate transcripts may be is still unknown.

It is difficult to quantify fold differences by visual inspection of an autoradiograph, as the quantification can be very subjective and different observers might not agree that one particular band is for example 2-fold more intense than another. In order to quantify these bands the autoradiographs

were densiometrically scanned to determine the intensity of each band and then given a numerical value. To correct for any variability in the amount of RNA loaded, the GnRH receptor value was divided by the β-actin internal control value. These results were then plotted on a histogram, with the average of the two control values taken as 100%. All other values were then expressed as a percentage relative to this value. The combined results from densiometric scanning of Northern blots from five independent experiments are shown in figure 3.7.

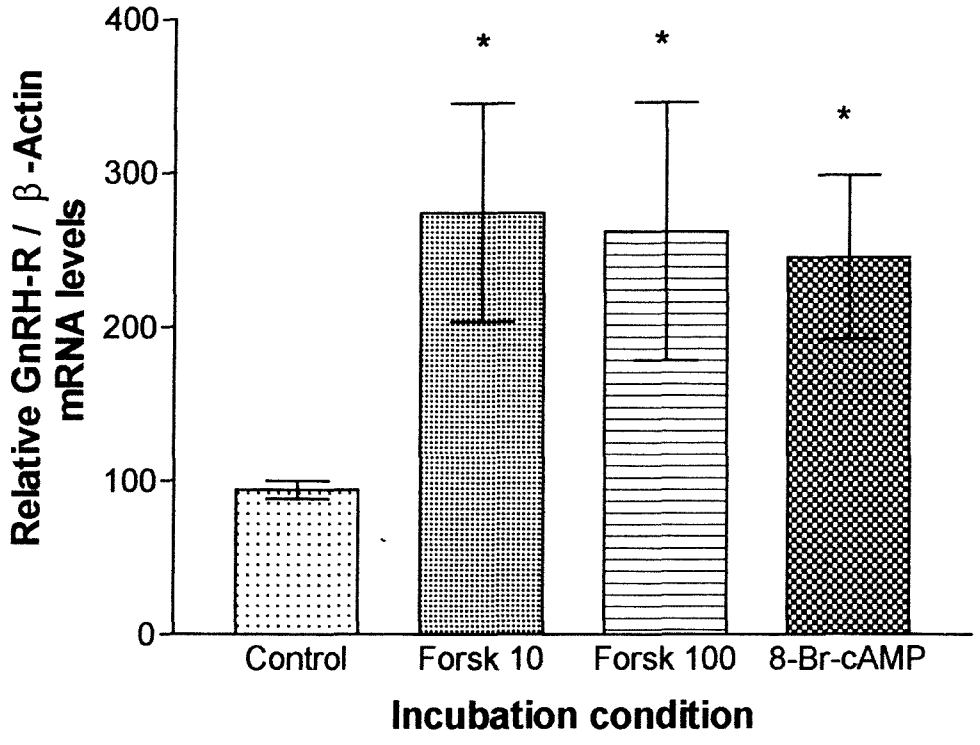


Figure 3.7: Effect of 8-Br-cAMP and forskolin on GnRH receptor mRNA levels in αT3 cells. The cells were incubated with 1 mM 8-Br-cAMP, 10 μM forskolin (Forsk 10), 100 μM forskolin (Forsk 100), for 18 hours, and some cells were not incubated with any test compounds (Control). The resulting autoradiographs (see figure 5) were densiometrically scanned and numerical values were given to each of the bands. In the case of the GnRH receptor the densities of the 4.5 and 1.8 kb bands were calculated and added together. The GnRH receptor values were divided by those for β-actin and the average of the two control samples were taken as 100 % and all other values were plotted as a percentage thereof. Standard errors are shown and the n value for the figure is 5. The * represent a P value of less than 0.05 ($P < 0.05$) relative to control.

The combined results of five independent experiments in figure 3.7 clearly show that activation of the PKA pathway increases expression of the endogenous GnRH receptor gene resulting in increased levels of mRNA. The results with 10 μM or 100 μM forskolin and 1 mM 8-Br-cAMP are very similar and they all caused an approximately 2.5-fold increase in the levels of GnRH receptor mRNA. The question remained however as to the mechanism of this effect: Was this increase the result of transcriptional control or did other mechanisms such as post-transcriptional modification also play a role? This was investigated in the next set of experiments.

B.) Does the PKA pathway effect the GnRH receptor gene at the transcriptional level?

The next set of experiments were performed to see if any PKA responsive elements are located in the 580 bp GnRH receptor promoter region, i. e. spanning from -580 to -1 relative to the ATG, and whether the stimulatory effect of the PKA pathway activation on the endogenous GnRH receptor gene occurs at the transcriptional level. This was done by transfecting αT3 cells with only the wildtype LG construct together with the β-gal construct, followed by incubation for various time periods with 10 μM forskolin, an adenylate cyclase activator. The results of four independent experiments are shown in figure 3.8. An example of original data from one such experiment is shown in table 3.2.

After a six hour incubation with 10 μM forskolin the promoter activity of the wildtype GnRH receptor had increased by 3-fold over uninduced levels (figure 3.8). The level of reporter gene expression after a twelve hour incubation with 10 μM forskolin showed a slightly higher increase than those of the six hour incubation i.e. a significant 3.5-fold increase in promoter activity over uninduced. It was very interesting to observe a time-dependent response to forskolin, as the cells treated for 18 hours showed no difference

in reporter activity compared to the control cells, while an even longer exposure of 24 hours decreased GnRH receptor promoter activity by 2-fold. The reason for this decrease is unknown, but some explanations could be that forskolin becomes toxic to the cells after such a long period or that the signalling pathways are desensitised and thus unable to respond to normal cellular signals, leading to a decrease in cell viability and a general slowdown of cellular processes. This would of course include transcription and translation and might explain why reporter activity is lower than in control cells.

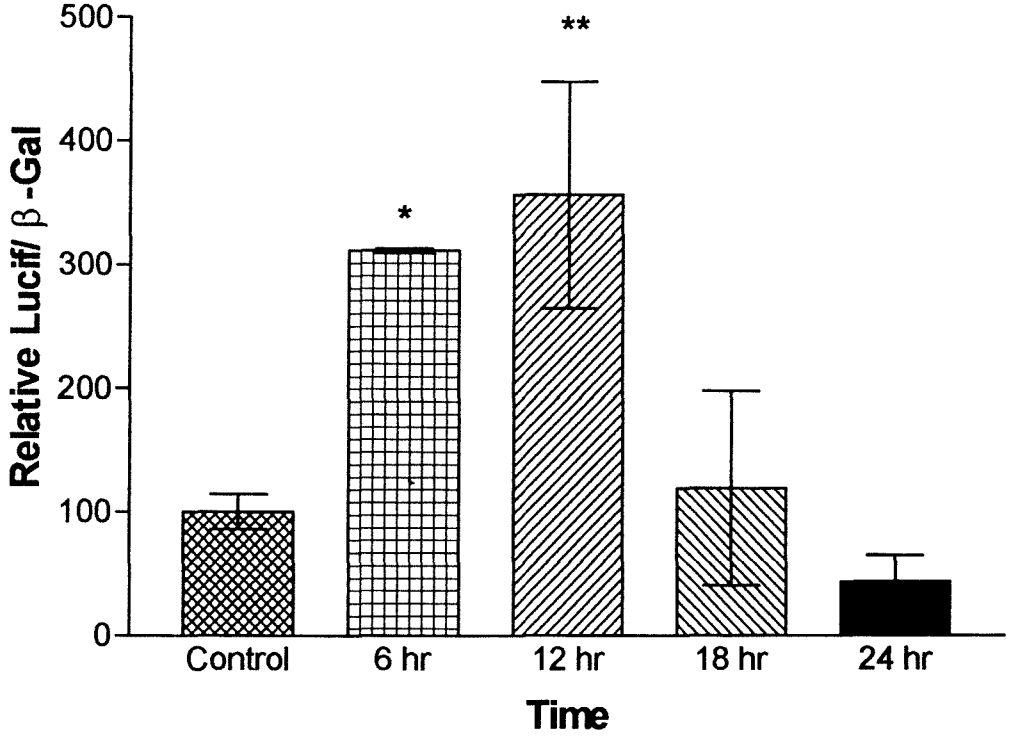


Figure 3.8: Incubation of wildtype GnRH-R reporter construct with 10 μM forskolin for varying times. αT3 cells were transfected with the wildtype (LG) GnRH-R reporter construct together with a β-Gal expression vector and incubated with 10 μM forskolin for various time periods (see table 2), before being harvested and assayed. Samples were transfected in duplicate and duplicate assays were also done on each sample. The luciferase values were divided by the corresponding β-Gal value to get normalised expression for each of the samples. The average of the wildtype (LG) was taken as 100% activity and all the other results from this experiment were calculated as a percentage thereof. Standard error bars are shown and the n value for the figure is 4. The * represent a P value of less than 0.05 ($P < 0.05$), while the ** indicate a P value of less than 0.01 ($P < 0.01$) relative to control.

Table 3.2: Example of original data from a single representative experiment on cells transfected with the wildtype GnRH receptor construct and incubated with 10 μM forskolin for various time periods.

		A	B	C	D	E	F	G	H	I
		Luciferase			β-Galactosidase			Lucif/β-Gal	% TO LG Ave	STDEV
		1	2	Mean	1	2	Mean			
Control	a	0.020	0.050	0.035	0.104	0.117	0.111	0.317	67.39	46.07
	b	0.100	0.090	0.095	0.155	0.150	0.153	0.623	132.5	
6 hr	a	0.150	0.140	0.145	0.098	0.099	0.099	1.472	313.2	2.510
	b	0.150	0.160	0.155	0.104	0.109	0.107	1.455	309.7	
12 hr	a	0.120	0.160	0.140	0.092	0.133	0.113	1.244	264.8	129.5
	b	0.250	0.070	0.160	0.082	0.070	0.076	2.105	447.9	
18 hr	a	0.050	0.010	0.030	0.078	0.090	0.084	0.357	75.99	195.6
	b	0.170	0.120	0.145	0.099	0.076	0.088	1.657	352.6	
24 hr	a	0.050	0.030	0.040	0.075	0.085	0.080	0.500	106.4	61.29
	b	0.010	0.010	0.010	0.083	0.133	0.108	0.093	19.70	

Columns A and B show duplicate luciferase assay results (i.e. 1 and 2) for an individual experiment with a given construct and "a" and "b" are the results from the duplicate cell incubation experiments for each construct. Column C contains the mean of columns A and B. The same applies for columns D, E and F but the values are from β-Galactosidase assays. Column G shows the normalised results, i.e. luciferase divided by β-Gal to normalise for transfectional efficiency. The average of the duplicate control samples, in this case LG, was then taken as 100% and all other results was calculated as a percentage of this value. This is contained in column H. Lastly column I contains the standard deviation between the duplicate values for each construct.

Taken together the results clearly show that PKA activation by forskolin stimulates transcriptional activity of the GnRH receptor promoter in a time-dependent fashion. This result strongly suggests that at least part of the increase in endogenous GnRH receptor mRNA shown in section 3A is in fact due to an increase in transcription. However after 18 hours of incubation with forskolin and/or 8-Br-cAMP, a 2.5-fold increase in endogenous GnRH-R mRNA levels was observed. At the same time point very little effect was seen on the GnRH-R reporter construct, which suggests that some post-transcriptional regulation may occur (see conclusions). The results also show that there are indeed PKA responsive elements located in the 580 bp GnRH receptor promoter fragment. The next experiment was set up to determine whether this PKA effect was mediated via the SF-1-like site 1 (the proximal site nearest to the ATG start codon).

4.) Role of SF-1-like site 1 in mediating the response to PKA activation

To determine whether site 1 contributed in any way to the stimulatory effect of the PKA pathway on transcription of the GnRH receptor gene, αT3 cells were transfected with either the wildtype (LG) or site 1 mutant (LGM1) constructs, together with the β-Gal construct, followed by incubation in the presence or absence of varying concentrations of forskolin for 12 hours. The results from 10 independent experiments can be seen in figure 3.9 and an example of original data from one experiment can be seen in table 3.3.

As in figure 3.8, a 10 μM dose of forskolin for 12 hours increased GnRH receptor promoter activity over uninduced or control (figure 3.9), but where the stimulatory effect was 3.5-fold in figure 3.8, here as shown in figure 3.9 the fold increase was only about 1.5-fold. This lack of reproducibility caused problems and forced the repetition of experiments to ensure that statistically

significant differences were obtained, as some experiments showed no effect with forskolin (see the section on optimisation for more information). The lack

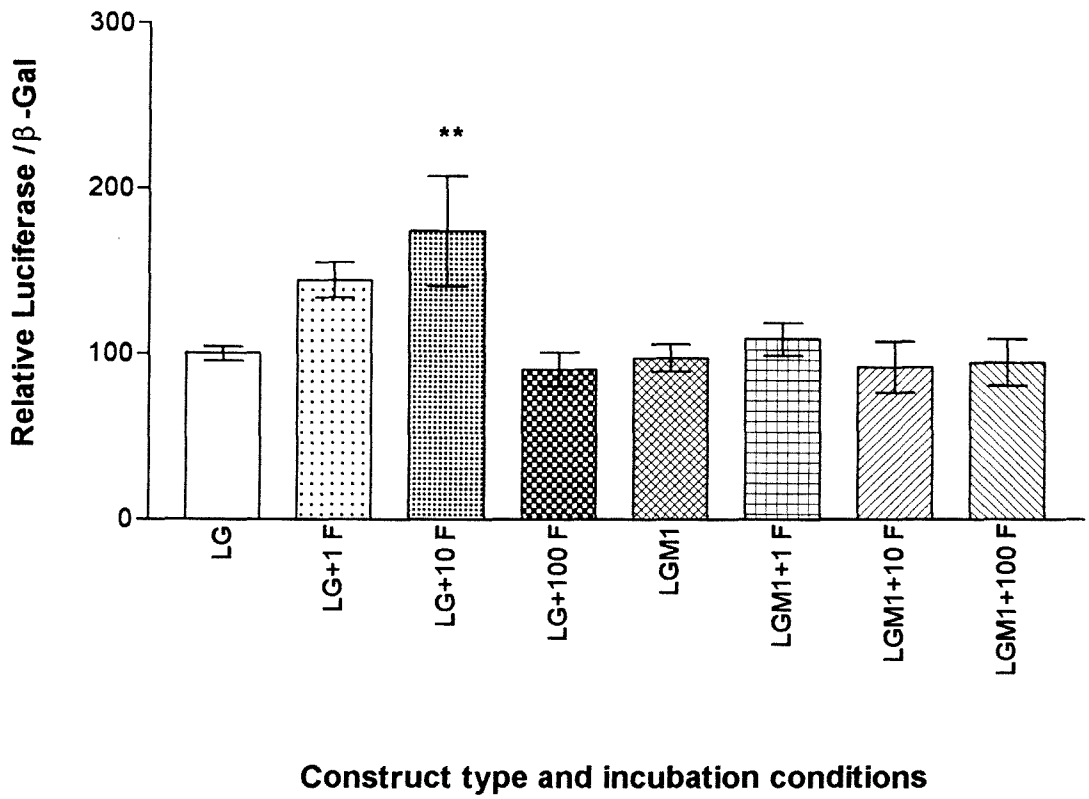


Figure 3.9: Effect of varying concentrations of forskolin on expression of two different GnRH-R promoter reporter constructs. αT3 cells were transfected with either LG or LGM1 plasmid and incubated with 0 (LG and LGM1), 1 (LG+1 F and LGM1+1 F), 10 (LG+10 F and LGM1+10 F) or 100 (LG+100 F and LGM1+100 F) μM of forskolin for twelve hours before harvesting. Cells were transfected in duplicate and duplicate assays were also performed. The luciferase values were divided by the corresponding β-Gal value to get normalised expression for each of the samples. The average of the wildtype (LG), with no forskolin, was taken as 100% activity and all the other results were calculated as a percentage thereof. Standard errors are shown and the n value is 10. The ** represent a P value of less than 0.01 ($P < 0.01$), relative to the control (LG).

of reproducibility could have been due to the inherent variability in the responsiveness of the cells between different experiments. Recent information [119] highlights the effect that the number of passages have on the characteristics of clonal cell lines. It would seem that the more times the

Table 3.3: Example of original data from a single representative experiment on cells transfected with the wildtype and site 1 mutant GnRH receptor constructs and incubated with 0, 1, 10 or 100 µM forskolin for 12 hours.

		A	B	C	D	E	F	G	H	I
		Luciferase			β-Galactosidase			Lucif/β-Gal	% TO LG Ave	STDEV
		1	2	Mean	1	2	Mean			
LG	a	0.177	0.199	0.188	0.041	0.041	0.041	4.558	92.48	10.62
	b	0.208	0.192	0.200	0.034	0.042	0.038	5.298	107.5	
LG+1µM	a	0.329	0.350	0.340	0.061	0.053	0.057	5.935	120.4	52.14
	b	0.349	0.361	0.355	0.037	0.038	0.037	9.569	194.2	
LG+10 µM	a	0.275	0.315	0.295	0.040	0.037	0.039	7.633	154.9	8.695
	b	0.226	0.223	0.225	0.033	0.031	0.032	7.027	142.6	
LG+100 µM	a	0.155	0.146	0.151	0.025	0.027	0.026	5.879	119.3	13.04
	b	0.141	0.108	0.125	0.027	0.024	0.025	4.970	100.9	
LGM1	a	0.148	0.145	0.147	0.022	0.025	0.023	6.328	128.4	13.41
	b	0.132	0.135	0.134	0.023	0.026	0.025	5.394	109.5	
LGM1+1µM	a	0.355	0.338	0.347	0.100	0.108	0.104	3.338	67.74	24.42
	b	0.342	0.354	0.348	0.071	0.068	0.069	5.040	102.3	
LGM1+10 µM	a	0.274	0.289	0.282	0.080	0.068	0.074	3.796	77.04	5.675
	b	0.329	0.309	0.319	0.066	0.086	0.076	4.192	85.06	
LGM1+100 µM	a	0.114	0.111	0.113	0.033	0.030	0.032	3.571	72.47	13.29
	b	0.117	0.115	0.116	0.050	0.038	0.044	2.645	53.68	

Columns A and B show duplicate luciferase assay results (i.e. 1 and 2) for an individual experiment with a given construct and "a" and "b" are the results from the duplicate cell incubation experiments for each construct. Column C contains the mean of columns A and B. The same applies for columns D, E and F but the values are from β-Galactosidase assays. Column G shows the normalised results, i.e. luciferase divided by β-Gal to normalise for transfectional efficiency. The average of the duplicate control samples, in this case LG, was then taken as 100% and all other results was calculated as a percentage of this value. This is contained in column H. Lastly column I contains the standard deviation of the duplicate values for each construct.

cells have been passaged, i.e. when confluent flasks are trypsinised to remove the adherent cells and subsequently plated out less densely into new flasks, the more undifferentiated they become. This partial revertment would cause the cells to lose some of their gonadotrope characteristics, such as the ability to respond to external or internal signalling pathways, like PKA, and result in a scenario where the GnRH receptor promoter constructs in these cells are no longer stimulated by PKA activators.

As shown in Figure 3.9 shows that 10 μM forskolin resulted in 1.5 fold increase reporter activity over uninduced. From figure 3.9 it can also be seen that even a low concentration of 1 μM forskolin appeared to increase reporter activity by 1.2-fold, although this effect was not statistically significant. However a forskolin concentration of 100 μM had no effect on the GnRH receptor promoter construct. The response to forskolin shows a similar trend to the one shown in figure 3.8 and the reasons for this might be the same as those discussed there. Indeed when one compares the β-Gal values from table 3.3, one can clearly see that the values are decreasing with an increasing concentration of forskolin. This strongly suggests a cytotoxic or desensitisation effect at high forskolin concentrations. The most interesting result from figure 3.9, however, is the set of results where αT3 cells, transfected with the mutated site 1 construct, LGM1, were incubated with various concentrations of forskolin. The most obvious difference between these set of results and those with the wildtype construct is the fact that the increase in reporter activity caused by 10 μM forskolin is not observed. The GnRH receptor promoter activity of the site 1 mutant incubated with 1 or 10 μM of forskolin is not significantly different to the uninduced control values. These results would be consistent with a role for a SF-1-like protein being responsible for mediating the stimulatory transcriptional effect of the PKA pathway on the GnRH receptor gene promoter via binding to the SF-1-like site 1 located 12 basepairs upstream of the ATG start codon.

Table 3.4: Example of original data from a single representative experiment on cells transfected with the wildtype and site 1 mutant GnRH receptor constructs and incubated with 0, 1, 10 or 100 µM 25-hydroxycholesterol for 12 hours.

		A	B	C	D	E	F	G	H	I
		Luciferase			β-Galactosidase			Lucif/β-Gal	% TO LG Ave	STDEV
		1	2	Mean	1	2	Mean			
LG	a	0.233	0.218	0.226	0.061	0.066	0.063	3.557	130.2	42.70
	b	0.101	0.100	0.101	0.045	0.060	0.053	1.907	69.80	
LG+1µM	a	0.395	0.418	0.407	0.071	0.073	0.072	5.634	206.2	21.32
	b	0.213	0.218	0.216	0.049	0.041	0.045	4.810	176.1	
LG+10 µM	a	0.097	0.077	0.087	0.030	0.033	0.032	2.762	101.1	25.52
	b	0.061	0.050	0.056	0.033	0.029	0.031	1.776	65.01	
LG+100 µM	a	0.008	0.010	0.009	0.032	0.025	0.028	0.317	11.62	4.053
	b	0.012	0.009	0.011	0.022	0.022	0.022	0.474	17.35	
LGM1	a	0.444	0.362	0.403	0.081	0.085	0.083	4.838	177.1	11.50
	b	0.376	0.382	0.379	0.070	0.073	0.072	5.282	193.3	
LGM1+1µM	a	0.222	0.191	0.207	0.067	0.053	0.060	3.450	126.3	30.88
	b	0.185	0.199	0.192	0.044	0.039	0.041	4.643	170.0	
LGM1+10 µM	a	0.036	0.024	0.030	0.053	0.021	0.037	0.803	29.40	73.37
	b	0.102	0.085	0.094	0.028	0.024	0.026	3.638	133.2	
LGM1+100 µM	a	0.014	0.024	0.019	0.011	0.018	0.014	1.315	48.13	14.27
	b	0.007	0.013	0.010	0.012	0.014	0.013	0.763	27.94	

Columns A and B show duplicate luciferase assay results (i.e. 1 and 2) for an individual experiment with a given construct and "a" and "b" are the results from the duplicate cell incubation experiments for each construct. Column C contains the mean of columns A and B. The same applies for columns D, E and F but the values are from β-Galactosidase assays. Column G shows the normalised results, i.e. luciferase divided by β-Gal to normalise for transfectional efficiency. The average of the duplicate control samples, in this case LG, was then taken as 100% and all other results was calculated as a percentage of this value. This is contained in column H. Lastly column I contains the standard deviation between the duplicate values for each construct.

5. Responsiveness of the GnRH receptor promoter to 25-hydroxycholesterol, the putative ligand for SF-1: Role of site 1

It has recently been proposed that 25-hydroxycholesterol (25-OHC) is a ligand for SF-1. To test this hypothesis αT3 cells were transfected with the

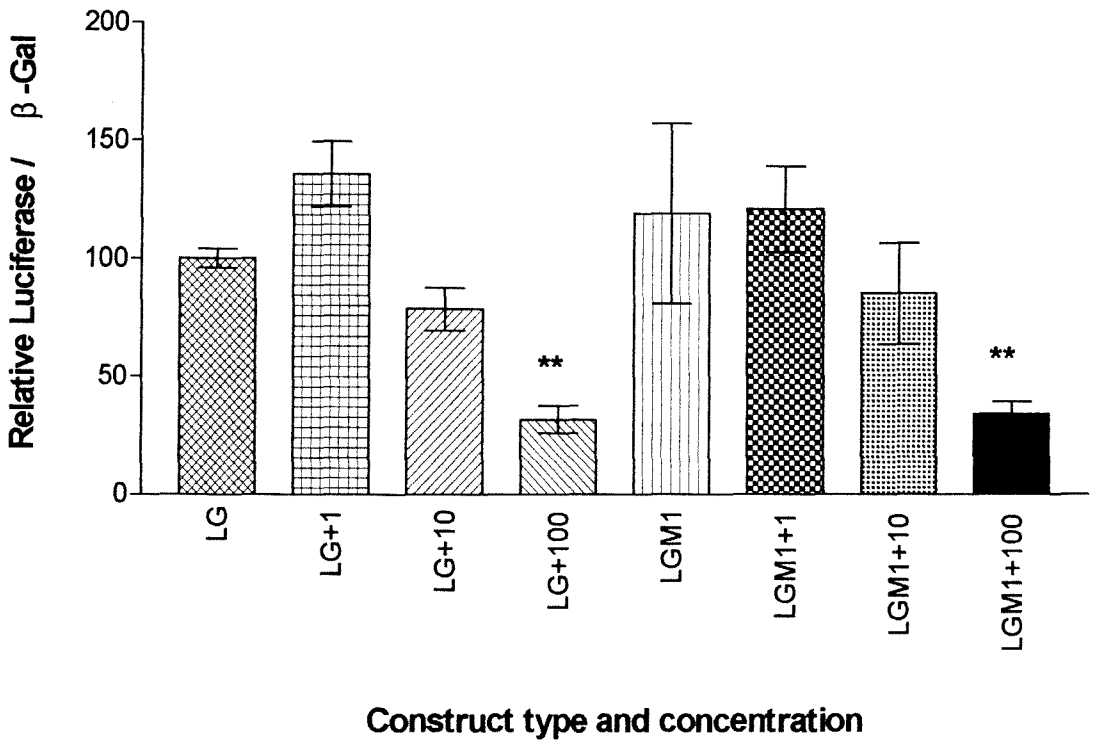


Figure 3.10: The effect of different concentrations of 25-hydroxycholesterol on two GnRH-R promoter reporter constructs. αT3 cells transfected with either wildtype or SF-1-like site 1 mutant constructs and β-galactosidase expression vector, were incubated with increasing concentrations of 25-OHC i.e. 0 (LG and LGM1), 1 (LG+1 and LGM1+1), 10 (LG+10 and LGM1+10) and 100 (LG+100 and LGM1+100) μM, for 24 hours before being harvested. Luciferase and β-Gal assays were performed and results expressed as luciferase over β-Gal. The average of the wildtype (LG), with no 25-OHC, was taken as 100% activity and all the other results were calculated as a percentage thereof. Standard errors are shown and the n value is 10. The ** represent a P value of less than 0.01 ($P < 0.01$) relative to LG.

wildtype and the mutated SF-1-like site 1 GnRH receptor promoter constructs, followed by incubation with varying concentrations of 25-OHC. Figure 3.10 shows the combined results from 10 independent experiments and the original results of one such experiment can be seen in table 3.4.

From figure 3.10 it is clear that no effect on transcription was observed with 1 and 10 μM 25-OHC for the wildtype construct, but there was a significant 1.5-fold drop in wildtype promoter activity when the cells were incubated with 100 μM 25-OHC. When the SF-1-like site 1 mutant construct was investigated, the same results were obtained as for the wildtype construct. It is interesting to note that the inhibiting effect of 100 μM 25-OHC occurred in both wildtype and site 1 mutant constructs, thereby suggesting that the binding of a SF-1-like protein to site 1 is not necessary for the inhibitory effect of 25-OHC. One possible explanation could be that 25-OHC becomes cytotoxic at these high concentrations and that this is what causes the decline in transcriptional activity seen at these concentrations. It is clear from table 3.4 that the absolute β-Gal values decrease with higher concentrations of 25-OHC, possibly because the cells are in fact dying. It is also possible that the 25-OHC response is mediated via SF-1-like protein, binding to site 2 rather than site 1.

6. Optimisation of assays

It can be seen from the transfection results in the previous section that for some series of data, the standard error for the results with different constructs showed a high degree of variability (e.g. figure 3.8). Error bars for some experiments were large, with a range of about 5 - 40% over the series of data. When the statistical analysis was performed for the whole range of experiments, those with large error bars decreased the ability to detect small differences less than about 1-fold with statistical significance, i.e. it was possible to detect differences of 1-3-fold but not smaller differences. One way of dealing with the problem was to repeat some experiments up to ten times (see figures 3.9 and 3.10). This was of course very time-consuming and used up large amounts of expensive reagents. The ideal situation therefore would have been to decrease experimental error to a minimum in order to be able to detect statistically significant smaller differences with a maximum of six

repeat experiments. The results in chapter 3, sections 1 -5, were, however, generated without the benefit of the optimised conditions and it was hoped that future researchers could benefit from these optimisation experiments.

There are many potential sources of variability during the performance of these experiments. The first source is in the fact that there is an inherent variability in the response. This means that some cells will react differently to certain experimental conditions. This possible source of variability cannot be eliminated and will always play a role in scientific experimentation. Other sources of potential variability could include experimental error e.g. differences in cell number, pipetting errors when adding DNA or test compounds to the cells, and other manipulation mistakes. Other areas of possible experimental error can be found in the assay protocols. The manual method of doing the luciferase assays involved the pipetting of the cell extracts into a plastic cuvette, as well as the pipetting of the luciferase assay substrate and assay buffer. The cuvette had to be vortexed to mix the different components and inserted into a luminometer where first a background reading had to be taken. The actual result had to be manually taken at a specific time after addition and the cuvette had to be carefully washed out before the next sample was introduced. All these different steps could introduce experimental error in the results.

When one looks at the actual data from various representative experiments, i.e. tables 3.1, 3.2, 3.3 and 3.4, it is easy to see where the biggest sources of error lie. It is useful to estimate the percentage error that the following three sources each contributed, i.e. the amount of error between experimental duplicates "a" and "b", the error between luciferase duplicates and the error between β-Gal duplicates. When the average percentage of experimental error in the four tables was estimated, it was clear that the experimental duplicates were the major source of error i.e. 40 - 60%. The estimated percentage error between β-Gal duplicates was around 10 - 30% and that of luciferase duplicates around 7 - 20%.

The main contributing factors to the large variability in experimental duplicates could either be error in plating out the cells or the inherent variability of the cells. Steps were taken as far as possible to ensure the most accurate pipetting. Cells were plated as carefully as possible by sucking up and down to avoid variability in the concentration of cell suspensions. Protein determination assays were performed on cell extracts (not shown) to determine if the plating of the cells was accurate. These protein determinations showed that the plating of the cells was indeed accurate, so that when results were expressed as luciferase over β-Gal over protein concentration, the error in the duplicates did not improve. It was thus concluded that most of the variability in the duplicates was due to inherent experimental variability.

It was thus decided to focus more attention on the luciferase and β-Gal assays. The laboratory recently acquired an automatic luminoskan machine that eliminated most of the manual pipetting steps during luciferase and β-Gal assays. The only pipetting step remaining was the pipetting of the cell extracts into a 96-well plate. The luminoskan would then automatically add the luciferase assay substrate, mix the contents of the wells in the plate, read the results after a defined time period and give a printout of the results. An added advantage was that the reaction could be scaled down by half, thus ensuring that the luciferase kit could be used for double the number of reactions as previously.

In figure 3.11 the result from a pilot study on reproducibility between the manual and luminoskan method can be seen. The same sample was read six consecutive times in the manual luminometer and it was also pipetted into six wells of a 96-well plate and automatically read. The average of the six samples was taken as 100% in each case and all other values were calculated as a percentage thereof. It is clear that the manual method of assaying resulted in a large error bar, i.e. the six readings differed by a

estimated 30% standard deviation, whilst the luminoskan method resulted in a dramatically decreased error bar with a standard deviation of about 2%.

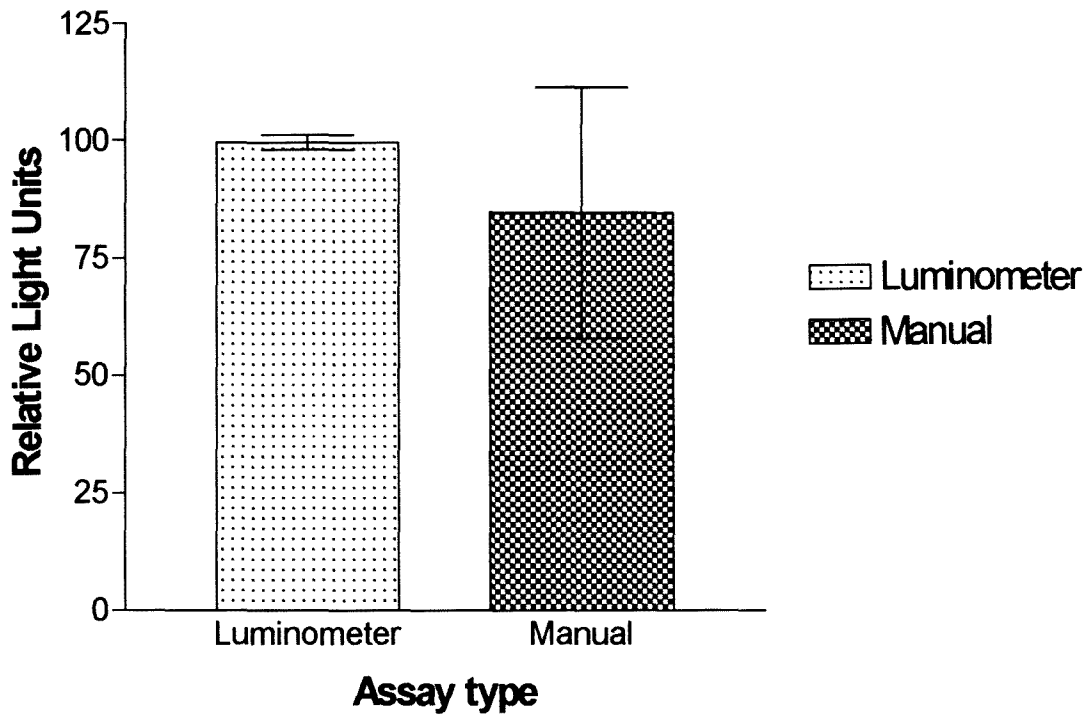
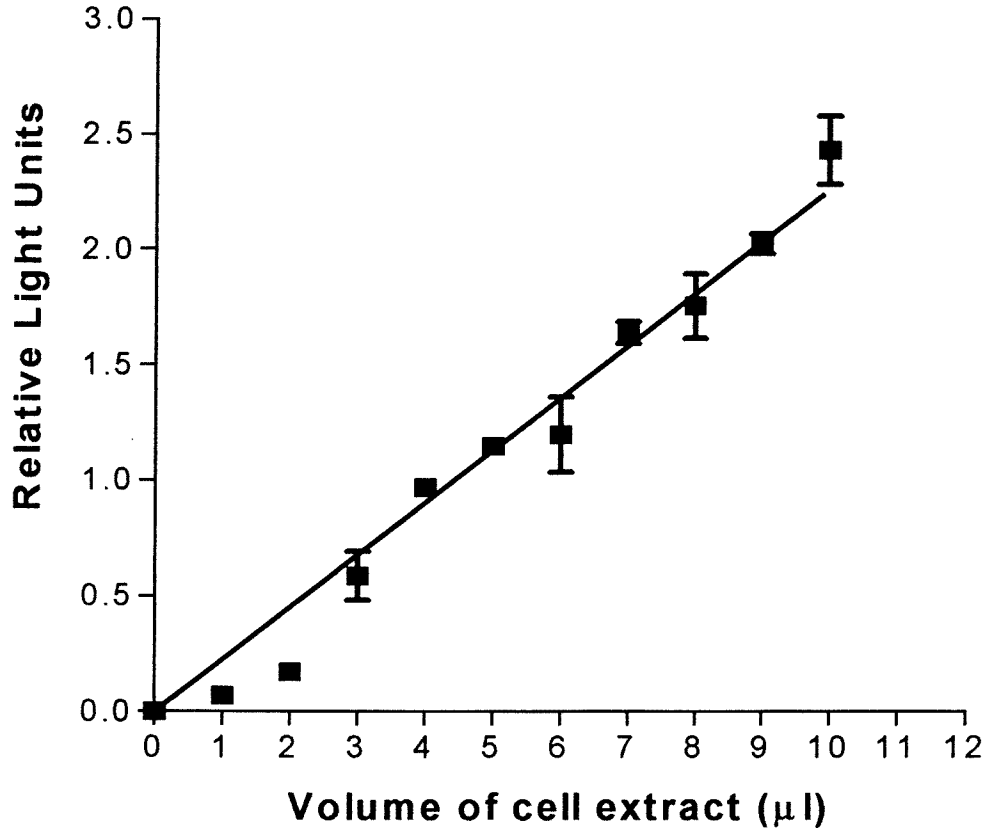
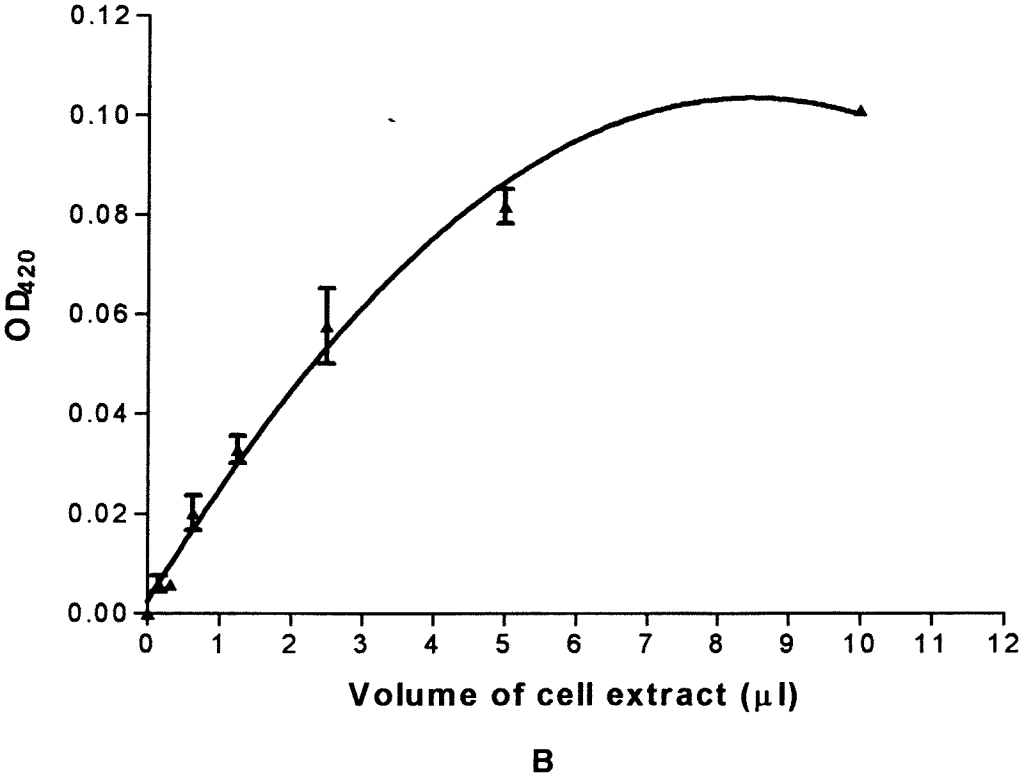


Figure 3.11: The difference in reproducibility between manual and luminoskan assay methods. Six consecutive readings of the same sample were performed in both the luminometer and with the manual method. The average of the results was then taken as 100 % and all values were expressed as a percentage thereof. Standard errors are shown and the n value is six.

The added advantage of the luminoskan was that both luciferase and β-galactosidase assays could be performed using chemiluminescence. Previously the β-Gal assays were done by a manual spectrophotometric method that also involved numerous pipetting steps and a colour reaction (see materials and methods). The β-Gal kit (Galacto-Star™, Tropix) used in the optimisation procedure is almost identical in methodology to the luciferase assay, but requires an additional incubation step and also utilises the automatic pipetting of the luminoskan machine. The assay makes use of a light-emission accelerator containing a polymeric enhancer [120].



A



B

Figure 3.12: Comparison of linearity and sensitivity between the chemiluminescent Galacto-Star assay (Panel A) and the manual spectrophotometric assay (Panel B). Various volumes

(μl) of cell extracts from αT3 cells used in previous experiments were assayed in duplicate with the two different methods and standard curves drawn.

A comparison between the linearity and sensitivity of the manual spectrophotometric assay and the chemiluminescent Galacto-Star™ assay can be seen in figure 3.12.

It is apparent from figure 3.12 that the Galacto-Star assay is linear from 0.5 to 2.5 relative light units (RLU) and throughout the range of cell extract volumes from 3 - 10 μl, whilst the manual assay is only linear between 0.005 - 0.06 OD_{420} and between 0.2 - 2 μl of cell extract before the line approaches a plateau. This seems to indicate that the manual method is more sensitive i.e. that it can be used to measure differences more accurately between smaller amounts of extract and that it is linear from 0.2 to 2 μl, whereas the Galacto-Star assay is less sensitive i.e. it cannot be used to measure the difference between 1 and 2 μl cell extract, but that it is linear over a wider range, 3 - 10 μl. This linearity means that if 3 μl of cell extract gave a reading of X RLU, then 6 μl would give a reading of 2X RLU. The reason for the short range of linearity found in the manual method might be due to the fact that the substrate might be limiting. Whatever the case may be, this result was somewhat disconcerting as some of the β-Gal assay values for the previous transfection experiments were performed with the manual method using 20 μl of cell extracts and obtaining OD_{420} readings much higher than 0.08. Most of the assays however had β-Gal values of less than 0.08. Even if some of the β-Gal values were inaccurate, it would not have effected the overall results, due to the large number of experiments that were done.

Since a major advantage of the luminoskan method over the manual assay method would hinge around the fact that the machine automatically pipetted assay substrate into wells of a 96-well plate, the reproducibility of the machine in this regard was tested. This was done by designating different areas on a 96-well plate and pipetting the same amount of cell extract into

each well in this block (see table 3.5) and then conducting a luciferase assay. The results are shown in figure 3.13.

Table 3.5: Designated blocks on a 96-well plate to test for automatic pipetting consistency

	1	2	3	4	5	6	7	8	9	10	11	12
A	**A**									**F**		
B						**D**						
C												
D												
E												
F					**C**							
G	**B**						**E**					
H												

As can be seen from figure 3.13, there are no statistically significant differences between the samples from the different areas on a 96-well plate. Note that this experiment not only assessed the automatic pipetting by the machine, but also the reproducibility of all parts of the assay, i.e. pipetting and detection in different areas of the 96-well plate. It can thus be concluded that the pipetting of assay substrate into different areas on the plate as well as the detection from different parts on the plate was highly reproducible.

In conclusion these results show that although the Galacto-Star β-Gal luminoskan assay method was not as sensitive as the manual β-Gal method in detecting differences between small amounts of cell extract, it had a much wider linear range of detection. The luminoskan method for both the Galacto-Star and luciferase method resulted in decreased experimental error, most likely by decreasing the amount of pipetting and handling steps. The wide linear range of the Galacto-Star assay would make it possible for future researchers to optimise their experimental conditions with the minimum

amount of experimental manipulation. Lastly it was shown that the luminoskan could automatically dispense and detect reproducibly in all areas of a 96-well plate. Thus future experiments could definitely benefit from the use of chemiluminescent luciferase and β-Gal assays performed in an automatically dispensing luminometer.

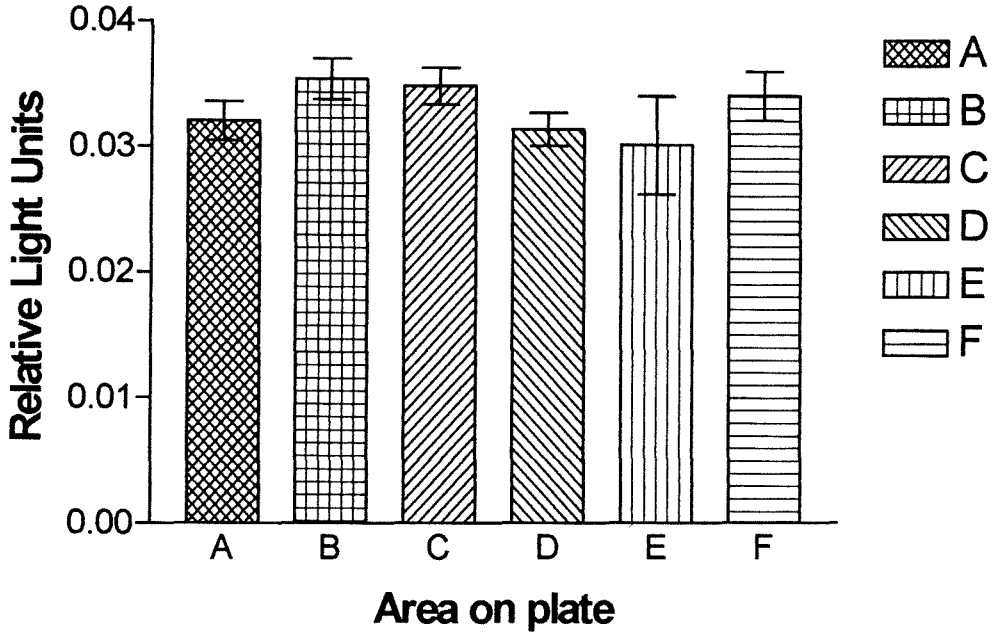


Figure 3.13: Comparison of different areas on a 96-well plate, with regard to the reproducibility of automatic pipetting and detection of the luminoskan machine. Cell extract was pipetted into various areas in a 96-well plate (see table 3.5) and the luminoskan then automatically dispensed luciferase assay substrate into target wells. The values of each area was used to generate a histogram with standard errors. The n values for each area differs i.e. the n value for area A, B and D is 6, C is 3, E is 4 and F is 9. The P value for this experiment was 0.1595.

CHAPTER 4

CONCLUSIONS

The promoter of the mouse GnRH receptor gene contains two potential SF-1 DNA-binding sites, i.e. at -247 and -14, relative to the ATG start codon. In this study, the role of steroidogenic factor-1 (SF-1) in the transcriptional regulation of the mouse gonadotropin-releasing hormone receptor (mGnRH-R) gene was investigated. SF-1 is an important regulator of steroidogenic gene expression and it is thought to act throughout the hypothalamic-pituitary/gonadal (HPG) and hypothalamic/pituitary/adrenal (HPA) axes. In general, various levels of transcriptional control exist, e.g. basal, cell- and tissue-specific regulation. In addition, controls are also exerted by hormonal and other signalling molecules which transiently modulate levels of gene expression via various intracellular signalling pathways like PKA or PKC. The mechanism of action and the role that SF-1 protein plays in all these levels of regulation is of great importance in elucidating the final picture of transcriptional control of the GnRH receptor gene.

SF-1 protein was initially thought to be the factor regulating the cell-specific expression of the gonadotrope genes, and SF-1 was identified as the protein that bound to the GSE in the α-subunit gene *in vitro* [6, 7]. It became apparent, however, that SF-1 protein was not the single determining factor that conferred cell-specific expression to these genes. It was later found that SF-1 protein, in conjunction with other tissue-specific DNA-binding factors, is involved in cell-specific basal expression of the GnRH receptor gene [62, 67].

Studies done on regulation of the various steroidogenic hormone genes, by SF-1 protein, also identified a different role for SF-1 protein, i.e. not only was SF-1 protein important in tissue-specific or basal expression of these genes, but in some cases it was found to mediate the effect of the PKA second messenger pathway on these genes [87, 90, 93, 95, 97 - 99]. This suggested

a pivotal role for SF-1 in gene regulation in the HPA/HPG axis and opened up the possibility that SF-1 might also fulfil this role on the GnRH receptor gene. This is, however, a controversial issue. There is no consensus in the literature about whether or not the GnRH-R gene is stimulated by activation of the PKA pathway. In addition, while results imply that SF-1-like site 1 and 2 are not involved in mediating a response to PKA [69], the role of sites 1 and 2 have not been directly investigated.

Another mystery still surrounding SF-1 protein is that of a possible ligand. A possible candidate is the oxysterol 25-hydroxycholesterol (25-OHC). This oxysterol was touted as the new ligand for SF-1 [111], but these experiments were performed in non-steroidogenic cells. However, other researches found that in a steroidogenic cell line, 25-OHC did not act as a ligand for SF-1 protein [113]. This issue is as yet still unresolved and has important implications for gene regulation in the HPA/HPG axis. If the ligand for SF-1 protein can be identified it would elucidate one more level of control over an already immensely complex system.

As stated above, the mGnRH-R gene promoter contains two SF-1-like sites. At the start of this study little information existed on the actual role of site 1. No known function was proposed for this site. It was shown to have no effect on basal or tissue-specific expression of the mGnRH-R gene [59]. SF-1 was proposed to bind to this site, but no binding data were reported in the literature. This site was also not directly tested for a role in a PKA response, but the general consensus was that it was not involved and played no important part in regulation of the mGnRH-R gene. On the other hand, site 2 was well studied and found to be necessary for gonadotrope specific expression of the mGnRH-R gene [59, 62, 67]. It was functionally shown, with antibody shifts, that this site could bind SF-1 protein *in vitro* [62]. However, this site was also not tested directly for a role in a PKA response.

It can thus be seen that, at the start of this MSc study, there were still a number of important questions to be answered regarding SF-1 protein and

the transcriptional regulation of the GnRH receptor gene. Firstly there was the question of whether SF-1 protein plays any role in basal expression of the GnRH-R gene. Secondly, another important question was whether the endogenous GnRH-R gene is regulated via the PKA pathway and whether SF-1 mediates this response. From the introduction section it is clear that the main regulator of this gene is its own ligand, GnRH. If the GnRH-R gene is in fact sensitive to the actions of the PKA pathway this could suggest that regulation of this gene by GnRH can occur through the PKA pathway, since there is evidence that GnRH activates the PKA pathway in animals [1, 22]. It is however also possible that other factors such as sex steroids or other signalling molecules that activate the PKA pathway can regulate expression of the GnRH-R gene. Another outstanding question was whether 25-OHC acts as a ligand for SF-1 in the non-steroidogenic αT3 cells.

Work previously done on mouse GnRH-R gene regulation in our laboratory identified the major transcriptional start site in αT3 cells and in mouse pituitary tissue at position -63 relative to the transcriptional start site (Hapgood, J.P. *et al.*, unpublished) and showed that the SF-1-like site 1 played no part in conferring tissue-specificity to the mGnRH-R gene [58]. It was also shown that a protein from αT3 cells could bind to this site. These findings were consistent with work done by other researchers [59]. The work in this thesis is a continuation of this study and focuses on other aspects of GnRH-R gene regulation.

The αT3 clonal mouse pituitary gonadotrope cell line was used as a model system in this study. The -580 to -1 bp promoter region, containing the two SF-1-like sites, of the mouse GnRH receptor (mGnRH-R) gene was investigated. Mutagenesis of these sites and subsequent transfection of reporter plasmid constructs into αT3 cells formed the basis of most of the experiments performed during this study. Levels of endogenous mGnRH-R mRNA were also studied and compared to results obtained with transfection experiments. Another important part of this study was to investigate the role

of the protein kinase A pathway in the regulation of the mGnRH-R gene and the role that SF-1 played in this regulation. The last area of investigation was the role of 25-hydroxycholesterol (25-OHC) in SF-1-dependent transcription of the mGnRH-R in αT3 cells.

Results obtained during this study showed that mutation of either SF-1-like site 1 alone or site 2 alone, had no effect on basal transcription of the mGnRH-R gene in αT3 cells. However simultaneous mutation of sites 1 and 2 led to a 50% reduction in transcriptional activity. The results with the individual mutations indicate that efficient basal expression of the GnRH-R gene requires the presence of at least one intact SF-1 site, occupied by SF-1 protein. The results imply that the occupation of both sites simultaneously by SF-1 is not necessary for basal expression. It is also conceivable that other nuclear receptors can bind to these mutated SF-1-like DNA-binding site and fulfil the role of SF-1 protein. Indeed this is the subject of a parallel study, by Hanel Sadie, done in this laboratory using electrophoretic mobility gelshift assays to investigate the proteins able to bind to the wildtype and mutated SF-1-like sites. Lastly it is thought that the two SF-1-like sites could somehow be positioned close together. This would facilitate the mechanism whereby any one of the two sites is important for basal expression of the mGnRH-R gene. Whether this coming together of the two sites takes place under physiological conditions is not yet known, but DNA looping and alterations in chromatin structure could be involved.

Previous work done in αT3 cells, [67] identified a tripartite enhancer in the mGnRH-R gene, consisting of a AP-1, SF-1 (i.e. site 2) and GRAS elements. These elements cooperate to regulate basal expression of the mGnRH-R gene and mutation of one single element alone led to a 60% decrease in transcriptional activity, whilst mutation of any two elements gave a 80% reduction in basal expression. Mutation of all three elements completely abrogated basal expression. The results obtained from the present study showing no effect when SF-1-like site 2 alone was mutated appear initially to

be inconsistent with the above mentioned findings. One possible reason for this could be that the construct used by Duval, D.L. *et al.* [67] and the construct used in the present study differ slightly in the 3' sequence of the SF-1-like site 1. The sequence of the SF-1-like site 1 in the construct used in this study was: TGTCCTTGGAGAAAGATCT and contained the 3'-immediate wildtype flanking sequence, whereas the Duval, D.L. *et al.* sequence was: TGTCCTTGGAGAAAtatgg. The differences in their construct compared to the one in the present study are indicated in lower case letters and the sequence shown to play a role in SF-1-DNA interaction is underlined. It is, however, not known whether these differences have any functional significance and apparent differences between results could have been due to other factors, like differences in culture conditions and assay types. It is interesting to note that simultaneous mutation of two SF-1 sites in the murine cytochrome P450 side-chain cleavage enzyme gene, led to a dramatic 7-fold decrease in basal transcription of this gene [90]. Unfortunately single mutations were not performed in this study and it is thus unknown whether basal transcription of this gene would also require the presence of only one intact SF-1 site as is the case in the mGnRH-R gene.

The results of the present study showed that activation of the PKA pathway led to an increase of endogenous mGnRH-R mRNA levels. Only one other study has reported an investigation into the effect of activation of the PKA pathway on endogenous GnRH-R mRNA levels. This was done by Alarid, E.T. and Mellon, P.L. (1995) [49], who found that forskolin caused a decrease in GnRH-R mRNA levels in αT3 cells. It is difficult to reconcile these results with the increases seen in the present study or the increases reported in the literature. The cell types used were the same, but the decreases were observed for time points of less than 12 hours. After 24 hours of incubation the mRNA levels were almost the same as for unstimulated cells. In the present study only the 18 hour time point was investigated by Northern blotting. It is possible that forskolin results in a decrease in the endogenous GnRH-R mRNA levels for the first few hours of incubation, after which the

levels start to rise, although it is unknown what the physiological reason for this could be. The mechanism for the increase seen in this study, however, was still unclear: Was this a case of transcriptional or post-transcriptional regulation? Transfection experiments are an ideal way of testing whether a certain response is in fact transcriptional or not, because it is possible to measure the activity of a certain gene promoter directly. Results from transfection experiments corroborated the mRNA results and showed that the PKA pathway could directly influence the promoter of the mGnRH-R in the αT3 cell line. In promoter transfection studies (fig. 3.8), it interesting to note that at 12 hours incubation time, with 10 μM forskolin, the GnRH-R promoter reached a maximum fold induction of 3.5 and that the fold induction for the 18 hour incubation was only 1.1-fold. The Northern blot mRNA studies on the other hand show that after 18 hours incubation time, with 10 μM forskolin, the maximum fold induction was 2.7-fold (fig. 3.7). This is extremely interesting and could mean that the effect of the PKA pathway is firstly only a transcriptional effect, up to 12 hours incubation time. At the 18 hr incubation time, the transcriptional effects may have ceased, but there may still have been a strong post-transcriptional effect in place. Perhaps a more likely explanation could be that the stability of the endogenous GnRH-R mRNA is greater than that of the luciferase reporter mRNA. This could have caused the luciferase reporter mRNA to be degraded by 18 hours, whilst there may still have been a high level of endogenous GnRH-R mRNA present at 18 hours. It is unknown whether such an effect could also take place *in vivo.*

The next obvious question was whether SF-1 plays any role in this PKA-dependent regulation, i.e. is this effect mediated via the SF-1-like DNA-binding sites in the mGnRH-R gene promoter? The present study showed that when the SF-1-like site 1 was mutated, the PKA-dependent increase in transcription was lost. Only site 1 was studied, however, due to time constraints. It would have been interesting to have had the results for both site 1 and site 2 in order to have the complete picture. Recent work done in GGH_3 cells by Maya-Nunez and Conn [69], identified a CRE element at

position -100 (relative to the ATG) in the mouse GnRH-R gene that is responsive to the PKA pathway and showed that mutation of this element abrogates the effect of PKA [69]. These results imply that sites 1 and 2 are not involved in mediating the PKA response, as they lost the effect if the CRE was mutated. These results at first appear to contradict the results of the present study. The direct involvement of sites 1 and 2 was, however, not tested by mutation or deletion of these sites. It can also be argued that GGH_3 cells are not pituitary gonadotrophs and therefore might not have the same transcriptional mechanisms as αT3 cells and might not even express SF-1 protein. If that is indeed the case, then the putative CRE might be inactive in αT3 cells, where the PKA-dependent response is mediated by SF-1-like site 1. Another possibility could also be that both the SF-1-like site 1 and the putative CRE work together to mediate the PKA response in αT3 cells. Evidence for such a co-operation was found in the rat aromatase gene promoter [85, 86]. This promoter also has a CRE about 80 basepairs upstream of the SF-1 site and it was found that both these elements were required for transcriptional activation of this gene by PKA. Interestingly, the fold increases found by Maya-Nunez and Conn [69] with forskolin treatment were in the 10% range. Such small increases would probably not have been detected with the assay systems used in this study. Thus in the present study, the response to PKA activation by an intact CRE and a mutated SF-1-like site 1 would not have been detected as statistically significant, even though there might have been a small 10% increase in promoter activity above uninduced control.

However, other work done in αT3 cells [60] showed that the PKA pathway is not involved in transcriptional regulation of the mGnRH-R gene, but that the PKC pathway fulfils this function. The authors further identified the AP-1 element, situated at -337 relative to the ATG, as important in mediating this response. Whilst these results may seem contradictory to results obtained in this study, it is important to remember once again that factors such as construct type (they also used the construct with the different 3'-flanking

sequences to the SF-1-like site 1, see above), the use of different culture conditions, i.e. different media, the use of antibiotics, the presence or absence of serum, as well as different number of passages for the αT3 cells, could result in variability in responses to cAMP and forskolin in αT3 cells in different laboratories. The finding presented in this thesis that the SF-1-like site 1 mediates the PKA response of the GnRH-R gene in αT3 cells is consistent with the role for SF-1 found in several other genes in other tissues, i.e. rat [85, 86] and human [87, 88] aromatase, rat [89, 90] and bovine [92] cholesterol side-chain cleavage, rat [93] and bovine [94] 17α-hydroxylase, human [95, 98], rat [96] and mouse [97] steroidogenic acute regulatory protein as well as the rat high density lipoprotein receptor [99].

The penultimate series of results generated during this study involved 25-hydroxycholesterol (25-OHC) and more precisely whether 25-OHC could effect SF-1-dependent transcription of the mGnRH-R in αT3 cells. No effect was found with 25-OHC at lower concentrations (1 μM and 10 μM) for either of the wildtype or site 1 mutant constructs. One interpretation of the results would be that since 25-OHC was not able to enhance SF-1-dependent transcription of the mGnRH-R gene in αT3 cells, then it is unlikely to be a ligand for SF-1 protein. It is, however, also possible that this decrease is a transcriptional effect and that it is mediated by a factor binding at a site other than site 1. Another possible explanation would be that the ligand, 25-OHC, was already present in these cells and addition of further 25-OHC had no effect on an already saturated system. Further experiments would be required in future to unravel these intriguing possibilities. However, at high concentrations of 25-OHC (100 μM) there was a 50% decrease in transcriptional activity for both the constructs. It is suggested that at these high concentrations, 25-OHC become cytotoxic and started to kill the cells. Visual inspection did not detect changes in the numbers and appearances of the cells at these high 25-OHC concentrations. Table 3.4 however clearly showed that the luciferase and β-Gal values of both the wildtype and site 1

mutant construct decreased with an increased 25-OHC concentration, consistent with a cytotoxic effect.

The last area that was investigated was the optimisation of the luciferase and β-Galactosidase assays. It was found that future researchers can benefit from using an automatic luminometer with automatic pipetting, together with chemiluminescent detection kits for both luciferase and β-Galactosidase. The luminoskan, with automatic pipetting, was able to decrease experimental error, normally in the 30% range for the manual luciferase assay, to about 2% for the same assay done in the luminoskan. An added benefit was the scaling down of the luciferase assay. Half the previous volume of reagent was needed, resulting in a doubling of the number of assays that could be performed with the luciferase detection kit. The chemiluminescent Galacto-Star β-Gal assays compared very well with the manual β-Gal assay, with a superior linear range from 3 - 10 μl, compared to the 0.2 - 2 μl of the manual method. However the manual method was more sensitive to differences between small volumes of cell extract. The wide linear range of the Galacto-Star assay could be very useful in optimising future experimental procedures with the minimum number of manipulations. The fact that the manual method was proven to be linear to only 0.06 OD units was worrying as some of the results generated via this method had values of more than 0.08 OD units. The linearity study for the manual β-Gal was only done later, together with the optimisation studies that were done on the automatic luminometer. With the benefit of hindsight it is clear that the linearity of the manual β-Gal method should have been performed at the start of the study and that it would have been wise to have repeated β-Gal assays on some of the samples with values larger than 0.08 OD_{420}, after dilution.

In summary, this study showed that the presence of one intact SF-1-like site in the promoter region of the mGnRH-R gene is necessary for basal expression of this gene, and that the absence of both SF-1-like sites leads to a 50% decrease in promoter activity. It was also shown that the PKA pathway

was able to increase mRNA levels of endogenous mGnRH-R gene in αT3 cells, and that this increase was most likely largely due to a transcriptional effect, but that evidence consistent with some post-transcriptional effects was also found. It was also shown that the SF-1-like site 1 mediated this PKA-dependent response. Lastly it was shown that in αT3 cells, the oxysterol 25-hydroxycholesterol, could not be shown to be a ligand for SF-1 protein. Optimisation experiments done during this study showed the advantages of using chemiluminescent detection kits in conjunction with an automatic dispensing luminometer.

One of the main implications of the findings in this study is to add to the increasing body of evidence that SF-1 protein plays a major role in the HPG axis. In this study, SF-1 protein was shown to regulate basal transcription of the GnRH receptor gene via either site 1 or site 2. It is interesting to note that a SF-1 site is conserved in the 5'-untranslated regions (5'-UTR) of the mouse (site 1), rat and human GnRH-R genes. This positional conservation is consistent with an important role for this SF-1 site in the transcriptional regulation of the GnRH-R gene. Since SF-1 is an orphan member of the nuclear receptor superfamily it is very important to identify its ligand, if indeed there is a ligand, in order to fully understand the signals that control the genes regulated by SF-1. In this study it was shown that it is unlikely that 25-OHC is indeed the ligand of SF-1 in αT3 cells. What was however clear from this study is that the PKA pathway can stimulate gene expression of the GnRH receptor gene in αT3 cells and that this effect is mediated via SF-1-like site 1. The important question of the mechanism by which SF-1 responds to PKA remains to be answered. Evidence for mechanisms involving indirect effects of PKA on SF-1 protein, obtained by others for some genes expressed in adrenal cell lines, include an increase in SF-1 mRNA levels, after treatment with forskolin [104], as well as post-translational modification of SF-1 protein [87]. Direct effects of PKA on SF-1 protein, shown by others for some genes expressed in adrenal cell lines, include direct phosphorylation of serine and threonine residues [93] or interactions via the activation function-2 (AF-2)

domain [94]. It is unknown which of these mechanisms take place in αT3 cells. The fact that the main regulator of this gene is its ligand, GnRH, raises the possibility that GnRH makes use of the PKA pathway and SF-1 protein to regulate transcription of its receptor gene. The potential also exists that the GnRH receptor gene can respond to multiple signals from the HPA/HPG axes e.g. ACTH, LH or FSH, or other factors that act via GPCRs or couple to Gs or Gi proteins. Whether receptors for these hormones occur in pituitary gonadotropes remains to be determined.

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APPENDIX A

Solutions

LB medium (Luria-Bertani Medium)

Per litre of deionized water add:

bacto-tryptone	10 g
bacto-yeast extract	5 g
NaCl	10 g

Sterilise by autoclaving

SOC medium

Per litre of deionized water add:

bacto-tryptone	20 g
bacto-yeast tryptone	5 g
NaCl	0.5 g

Add 10 ml of a 250 mM solution of KCl. Adjust the pH to 7.0 with 5 M NaOH. Adjust the volume to 1 litre and sterilise by autoclaving. Just before use add 5 ml of a sterile solution of 2 M $MgCl_2$, and 20 ml of a sterile 1 M solution of glucose.

SOB Medium

SOB medium contains the same nutrients and salts as SOC (above), but lack the glucose.

20 X SSC Solution

Dissolve 175.3 g NaCl and 88.2 g of sodium citrate in 800 ml water. Adjust the pH to 7.0 with addition of a few drops of 10 M NaOH. Adjust the volume to 1 litre and dispense into aliquots. Sterilise by autoclaving.

TE buffer pH8

10 mM Tris.HCl (pH 8)

1mM EDTA (disodium salt) (pH 8)

10 X Trypsin Solution

Per 500 ml deionized water:

EDTA	1 g
NaCl	40 g
KCl	1 g
NaH_2PO_4	5.75 g
KH_2PO_4	1 g
Glucose	1 g
Trypsin (BDH)	6.25 g

Adjust the pH to 7.2-7.4. Make up the volume to 500 ml with water. Filter sterilise and store at -20 °C.

10 X MOPS Buffer

Morpholinopropanolsulfonic acid	41.86 g
Sodium Acetate	4.10 g
EDTA (disodium salt)	3.72 g
DEPC-treated H_2O	to one litre

Adjust the pH to 6.0

2 X HeBS (HEPES Buffered Saline)

Dissolve 1.6 g of NaCl, 0.074 g of KCl, 0.027 g of Na_2HPO_4, 0.2 g of dextrose and 1 g of HEPES in 90 ml of deionized water. Adjust the pH to 7.05 and make the volume up to 100 ml. Sterilise through 0.22 micron filter and store in aliquots at -20 °C.

APPENDIX B

Plasmid constructs